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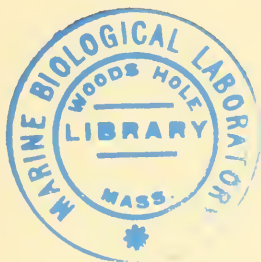




# The Initial Effects of Ionizing Radiations on Cells



# The Initial Effects of Ionizing Radiations on Cells



*Edited by*

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## CONTENTS

LIST OF CONTRIBUTORS.....	v
INTRODUCTION.....	xi
Opening Address. By N. M. SISSAKIAN.....	1
The Nature of the Initial Radiation Damage at the Sub-Cellular Level. By P. ALEXANDER AND Z. M. BACQ.....	3
Mechanisms Involved in the Initiation of Radiobiological Damage in Aerobic and Anaerobic Systems. By L. H. GRAY.....	21
Action of Radiation on Proteins and Nucleic Acids in Solution and at Interphases. By A. G. PASSYNSKY.....	45
Electron Spin Resonance (ESR) Investigations on Radiation-Induced Chemical Effects in Biological Species. By L. A. BLUMENFELD AND A. E. KALMANSON.....	59
The Action of X-Rays on Intracellular Bacteriophage Formation. By F. HERČÍK.....	69
The Action of Ionizing Radiation on the Cellular Synthesis of Protein. By ERNEST POLLARD.....	75
Chemical Species Induced by X-Rays in Cells and their Role in Radia- tion Injury. By E. L. POWERS.....	91
Fluorescence Studies of the Changes Undergone by Nucleoproteins and their Derivatives in Irradiated Cells. By M. N. MEISSEL, E. M. BRUMBERG, T. M. KONDRATJEVA AND I. J. BARSKY.....	107
A Discussion of Some Immediate Effects of X-Irradiation on Living Cells. By BARBARA E. HOLMES.....	131
Radiation-Induced Disturbances of the Lipids of Cellular Micro- structures. By N. N. DOEMIN AND V. D. BLOKHINA.....	141
The Significance of Free Deoxyribonucleotides in Radiation Damage. By J. SOŠKA, L. BENEŠ, V. DRÁŠIL, Z. KARPFEL, E. PALEČEK AND M. SKALKA.....	153
Biochimie et Radiobiologie du Noyau Cellulaire. By M. ERRERA.....	165
On the Mechanism of Lethal Action of X-Rays in <i>Escherichia coli</i> K12. By H. MARCOVICH.....	173

Damage to the Reproductive Capacity of Human Cells in Tissue Culture by Ionizing Radiations of Different Linear Energy Transfer. By G. W. BARENDSEN. ....	183
Phosphate Metabolism in the Nucleus. By L. A. STOCKEN. ....	195
Initial Steps in Radiation Damage to Chromosomes and Means of Preventing this Effect. By ALEXANDER HOLLAENDER. ....	201
On the Mechanism of Inhibition of Cell Division Induced by Ionizing Radiation. By A. V. LEBEDINSKY, V. M. MASTRYUKOVA AND A. D. STRZHIZHOVSKY. ....	211
The Biochemical Mechanism of the Disturbance of Cell Division by Radiation. By A. M. KUZIN. ....	223
The Role of Cellular Damage in the Mammalian Radiation Syndrome. By E. Y. GRAYEVSKY, I. M. SHAPIRO, M. M. KONSTANTINOVA AND N. F. BARAKINA. ....	237
Studies on Enzymes and Yeast Cells with Accelerated Heavy Ions. By CORNELIUS A. TOBIAS, TOR BRUSTAD AND THOMAS MANNEY. ....	257
The Rhythm of Oxidative Processes and its Disturbance under the Action of Radiation. By G. M. FRANK AND A. D. SNEZHKO. ....	269
Immediate Reactions of Nerves and Muscles to Ionizing Radiation. O. HUG AND H. J. SCHLIEP. ....	287
Mechanisms of Chemical Radiation Protection. By Z. M. BACQ AND P. ALEXANDER. ....	301
Kinetics of Primary Reactions and Chemical Protection. By V. N. TARUSOV. ....	315
GENERAL DISCUSSION. ....	327

## INTRODUCTION

In this volume are published the papers and discussion of the International Symposium on Primary and Initial Effects of Ionizing Radiations on Living Cells—the effects that underlie the biological effects of radiation. The Symposium was held in Moscow in October 1960 and was organized by the Academy of Sciences of the U.S.S.R., under the auspices of UNESCO, and co-sponsored by IAEA. The President of the Symposium and the organizer of the very interesting general discussion was an outstanding Belgian scientist, Professor Z. M. Bacq, who is a foreign member of the Academy of Sciences of the U.S.S.R.

The symposium was of round table type. The scientists from different countries, invited personally by the Academy of Sciences jointly with the Department of Natural Sciences of UNESCO, are very well known for their important achievements in the field of radiobiology at the molecular, subcellular and cellular level. In the papers presented at the symposium were given not only their latest results but also some general points of view on the state of the problem as a whole. The scientists discussed the most urgent and yet unsolved problems so, besides the reports and discussions after each report, a certain period of time was allotted to general discussion.

Paying attention to this fact it was decided to shorten the prolixities and repetitions in the publication of the Proceedings of the Symposium and to publish not only the texts of the reports but also the discussion referring to separate reports, as well as the whole of the general discussion. In publishing the papers a number of comments, that were not originally made (for lack of time), but were presented by the participants in written form, are included in that general discussion. This seemed expedient since the material supplemented the interesting discussion. In order to make the discussion more wide and lively not only the participants but also the guests at the Symposium took part.

The "round table" type of symposium has certain advantages. It provides close contact and a free and easy conversation among the limited numbers of participants. It goes without saying that, as well as the official discussion, the unofficial discussion, during the intervals and after the sessions were formally closed, much more often compared with that of more crowded conferences. The results of such meetings invisibly, in, as it were, a "latent" fashion, affected the points of view

which were expressed by the participants during the final general discussion.

By the extremely wide contemporary development of each field of knowledge, by the application of the most diverse techniques, and by study of the processes with different objects, we must not be disappointed when there seems at first sight to be nothing in a report or discussion that would appear to solve the problem, or give the impression of a major qualitative leap forward. Each important scientific problem is being solved step by step through the laborious investigation of many tens and hundreds of scientists, when the data are accumulated and concepts develop. Therefore we think that the summing-up of the modern points of view and of the state of the problem, with the simultaneous distinct demonstration of new tendencies and ways of approach, is the most important and principal result of such a symposium; these can be found in the published material. Undoubtedly, too, the published material will, in many respects, be a "stimulus" for a further development of science in the field of primary processes produced by radiation, and by the cells-reactions to irradiation.

I should like to express my heartfelt gratitude to all the participants for the efforts they made. I also consider it my duty to express my cordial thanks to all who took part in preparing the materials for publication, and especially, for their hard work in preparing the debate and general discussion. Simultaneously with the present edition the same material is being published in the Russian language by the U.S.S.R. Academy of Sciences.

G. M. FRANK

## OPENING ADDRESS

*Delivered on behalf of the Academy of Sciences of the U.S.S.R. by  
Academician-Secretary of the Department of Biological Sciences,  
U.S.S.R. Academy of Sciences*

N. M. SISSAKIAN

Ladies and Gentlemen,

Dear Colleagues, I welcome you on behalf of the U.S.S.R. Academy of Sciences and the U.S.S.R. UNESCO Committee.

We are happy to see our guests, eminent scientists from many countries, who have come to discuss interesting and outstanding problems. In you I also greet the important international organizations, UNESCO and the International Atomic Energy Agency which, in co-operation with the U.S.S.R. Academy of Sciences, have sponsored the present symposium.

We are glad to see here the representatives of these organizations. In UNESCO and the International Atomic Energy Agency are combined the efforts of many nations in working out important problems of science, in spreading culture and knowledge and in the use of atomic energy for the good of mankind.

Soviet scientists are eager to establish international co-operation with the aim of mutual understanding, peace and friendship among nations. They are willing to use all their resources and energy to organize international meetings and contacts, which are so important for the progress of science.

The rapid development of science in our age, the interconnection of a number of disciplines and the vast data accumulated require that science should be organized on quite new lines. Individual scientists cannot achieve spectacular results as they used to in the last century. Large teams of research workers in scientific institutions and universities cannot exist in isolation. We require constant exchange of opinions, information, data and techniques: during these contacts and discussions new ideas are born and new ways of investigation are discovered. The level of modern science and the rate of its development make international meetings especially necessary.

The present twentieth century is justifiably called the age of atomic energy. A new, exceptionally powerful agent has entered the life of men and advanced the solution of a number of most important problems. At the same time mankind is facing a number of highly important



theoretical and practical problems. The use of atomic energy has stimulated the rapid development of a new biological discipline radiobiology.

This science aims to establish the laws of the action of radiation on living organisms. It is connected with the solution of essential tasks in most complex fields of human activity, medicine, agriculture and industry. The use of this energy is connected with the discovery of the most effective methods of radiobiology, the breeding of new species of plants and strains of useful micro-organisms. One of the most important tasks of this new science is, no doubt, that of protecting those who come into direct contact with this agent, and their progeny, from the damage of ionizing radiation.

To solve these problems, it is first of all necessary to know the initial stages of the reaction developing from the interaction of a living organism with ionizing radiation. A deep scientific knowledge of the processes initiated by every kind of radiation is indispensable. These problems require the close co-operation of experts in various branches of science: physicists, biologists and physicians. Radiobiology is a border discipline: formed on the cross-roads of physics, chemistry, biology and medicine, it makes wide use of approaches and techniques peculiar to these sciences.

The present symposium is devoted to the important problems of the primary action of ionizing radiations on biological substrata and on the cell. I am deeply convinced that as a result of the work of the present meeting of eminent scientists, at which so many outstanding radiobiologists are present, we shall be able to sum up year-long researches on these problems and to map out new paths along which radiobiology will develop. The development of radiobiology is most closely connected with the very important problems of our time, the protection of mankind from the damaging effect of the production and testing of nuclear weapons. Radiobiologists are aware, more than anybody else of the damage caused by even a minimal dose of radiation not only to the present generation, but to the future ones as well.

Therefore, the voice of radiobiologists, warning of the danger of nuclear tests is specially important in the strengthening of peace on earth and in the establishment and development of friendship among nations. I should like to wish success to the work of the members of the present symposium and to express the hope that it will enhance friendship and creative co-operation between the scientists of our various countries.

# THE NATURE OF THE INITIAL RADIATION DAMAGE AT THE SUB-CELLULAR LEVEL

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## SUMMARY

A detailed investigation of the changes produced in DNA, when this is exposed to ionizing radiations under a variety of conditions, indicates that radiochemical damage to DNA is unlikely to be the primary event which initiates the processes which eventually result in the death of irradiated cells. Further support for this view is derived from the fact that the sensitivity of the nucleoprotein when irradiated within the cells is approximately the same for cells of widely different radiosensitivities. Attention to the non-genetic components of the cell is indicated by the observation that treatment with iodoacetate before irradiation can increase the radiosensitivity of cells seven-fold. Studies of mouse leukaemia cells in tissue culture indicate that chromosome breakage is not an important anatomical lesion for the death of these cells. The hypothesis that interference with the sub-cellular fine structure of the cell constitutes an important primary lesion is discussed.

As the radiation passes through the cell it deposits energy, part of which is used up to produce ionizations which initiate chemical reactions that cause some of the cell constituents to be chemically altered. There are several steps between the ionization of a molecule and its final chemical state (see "repair" of molecule in this symposium, p. 301) but the time taken for this process is, *in vivo*, a small fraction of a second.†

† In model experiments, chemical changes have been observed hours after the irradiation is complete. Usually this is due to the fact that solids are treated in which the movement of molecules is limited and radical combination is slowed down. This situation may be encountered in dry biological preparations such as spores or seeds, but does not occur in wet cells. This explains why radicals can be detected by electron spin resonance in dry systems (including seeds, etc.) but no such signal can be observed in irradiated wet cells.

Most of these chemical reactions are biologically trivial and do not harm the cell. But some of these occur at vital points and act as a focus for the development of damage by subsequent cell processes. These we have called the *Initial (Chemical) Lesions*, and their nature forms the subject of this paper. We have very little definite information about the early chemical events. The U.N. Scientific Committee on the effects of atomic radiation (UNSCEAR) concluded in its report (1958): "The nature of the initial step of radiation damage remains to be determined."

After a certain period, the duration of which depends on the intensity of the metabolism, biochemical lesions can be observed and these lead to anatomical lesions (i.e. biological end effects). At the cellular level the immediate biological effects can be conveniently classified into (i) physiological; (ii) cell lysis or interphase death; (iii) delayed death often requiring mitosis before becoming manifest. It is improbable that the same radiochemical reactions initiate all these different biological effects. Also, a number of these may act in conjunction to produce one end effect, such as mitotic death, and their relative contribution may vary from one type of cell to another.

Numerous methods are suitable for approaching the problem of the nature of the initial lesions, such as various biophysical techniques, biochemical techniques, comparison between the effects of *in vivo* and *in vitro* irradiation of key molecules (enzymes, proteins, RNA, DNA, etc.) and the study of the mechanisms of action of chemical protectors. A study of the physiological effects<sup>†</sup> of radiations may be particularly useful in this connection since they occur almost immediately after irradiation and the initial lesion must therefore be more closely related to the biological effect observed than in the case of such phenomena as cell death, chromosome abnormalities, etc., where many hours of active metabolism intervene.

<sup>†</sup> It has become clear within the last few years that numerous disturbances induced by ionizing radiations occur almost immediately after irradiation (i.e. seconds to minutes) and must be attributed to an interference with the physiological function of nerve fibres and cells, and to changes in membranes. Particularly striking demonstration of such immediate effects have been provided by Brinkman and Lamberts (1960) and by Hug (1960) with snails, echinoderms and isolated mammalian organs. Very rapid effects on the retina of vertebrates have also been reported at the earlier UNESCO symposium at Venice (1959) the proceedings of which have been published as Supplement No. 1 (1960) to the *International Journal of Radiation Biology*. All these changes are physiological in the sense that they are characterized by extremely rapid repair and are therefore very dose-rate dependent and may have to be studied while the irradiation is going on. Very rapid changes in the transport of the ions have been encountered following irradiation of plant roots (see Bacq and Alexander, 1961) but we know of some negative unpublished experiments with animal membranes.

It is not a paradox to say that for certain biological systems, the ionizing radiation at small doses or dose-rates may be a stimulus comparable to an electric current or visible light and that no permanent lesion is inflicted on the organism.



## DOES DAMAGE OF A VISIBLE STRUCTURE REGULARLY PRECEDE CELL DEATH?

The problem of finding the nature of the initial chemical lesion in cell death would be simplified if this effect could be associated with damage of a particular organelle, such as the chromosome. No generalization is, however, possible and not even the relative importance of the cytoplasm and the nucleus has been resolved. In certain cells (insect eggs for instance) the lesion of the nucleus seems to be all important but in other cells (amoebae, amphibian ovarian eggs) the contribution of the cytoplasm is certainly very great (for ref. see Bacq and Alexander, 1961).

While some cells such as lymphocytes, spermatogonia and oocytes are killed outright (i.e. interphase death) by a few hundred rads, most mammalian cells need rather large doses for this to occur and, in general, they are more sensitive to mitotic death: that is, they will divide once or twice before division stops though the cells continue to grow in size.

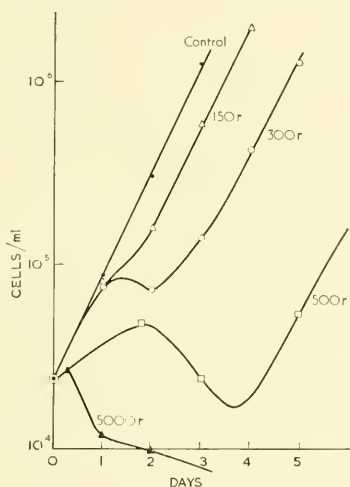


Fig. 1.—Effect of X-rays (220kV at 300 + min) on growth of mouse leukaemia cells in tissue culture (Alexander and Mikulski, 1960) (The cells were in suspension and fully oxygenated when irradiated).

Figure 1 illustrates this effect for mouse leukaemia cells in tissue culture. Until the cell number has doubled, no effect of radiation with doses of 300r or less was seen, then cell division ceases (with 300r in case of 90 per cent of the cells) and the damaged cells increase in size. (Alexander and Mikulski, 1960). The majority grow to about double the normal volume but about a quarter of them continue to grow and form

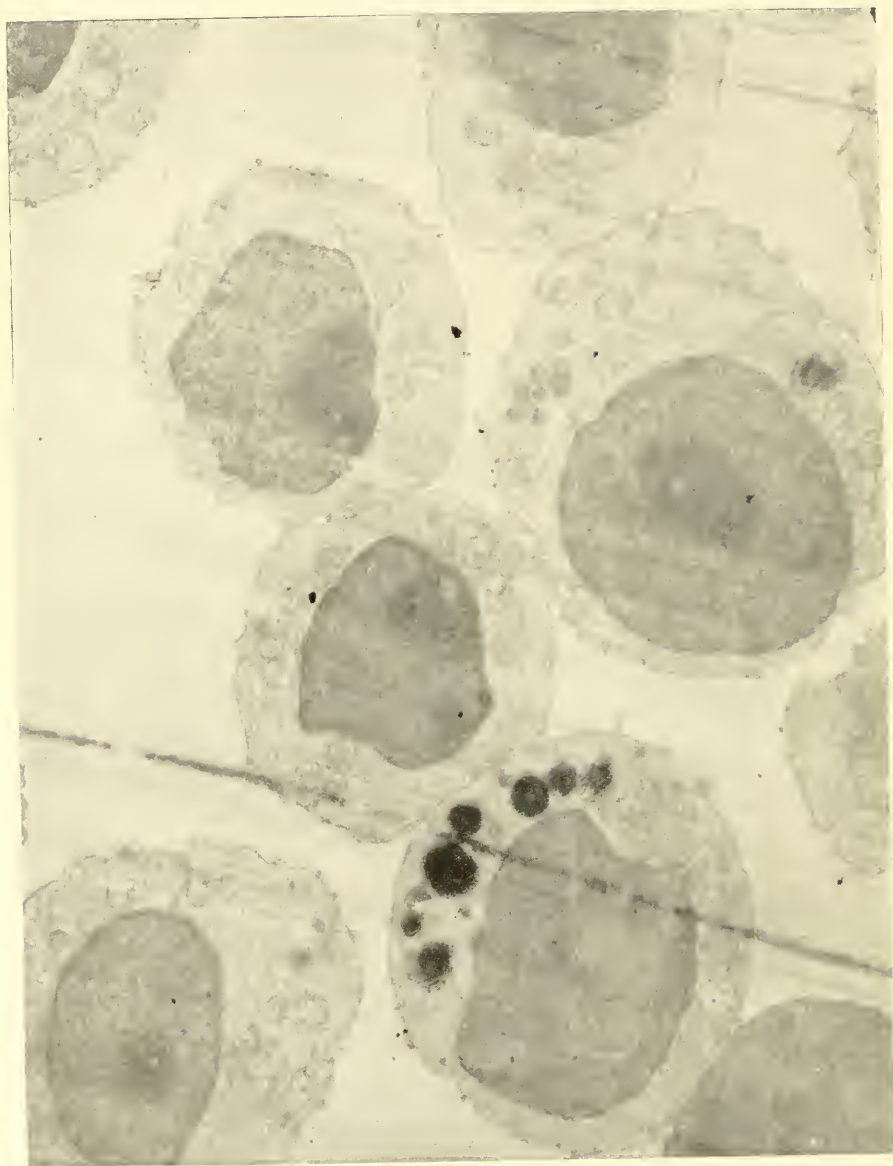


Fig. 2. — Electron photomicrograph of mouse leukaemia cell grown in tissue culture.

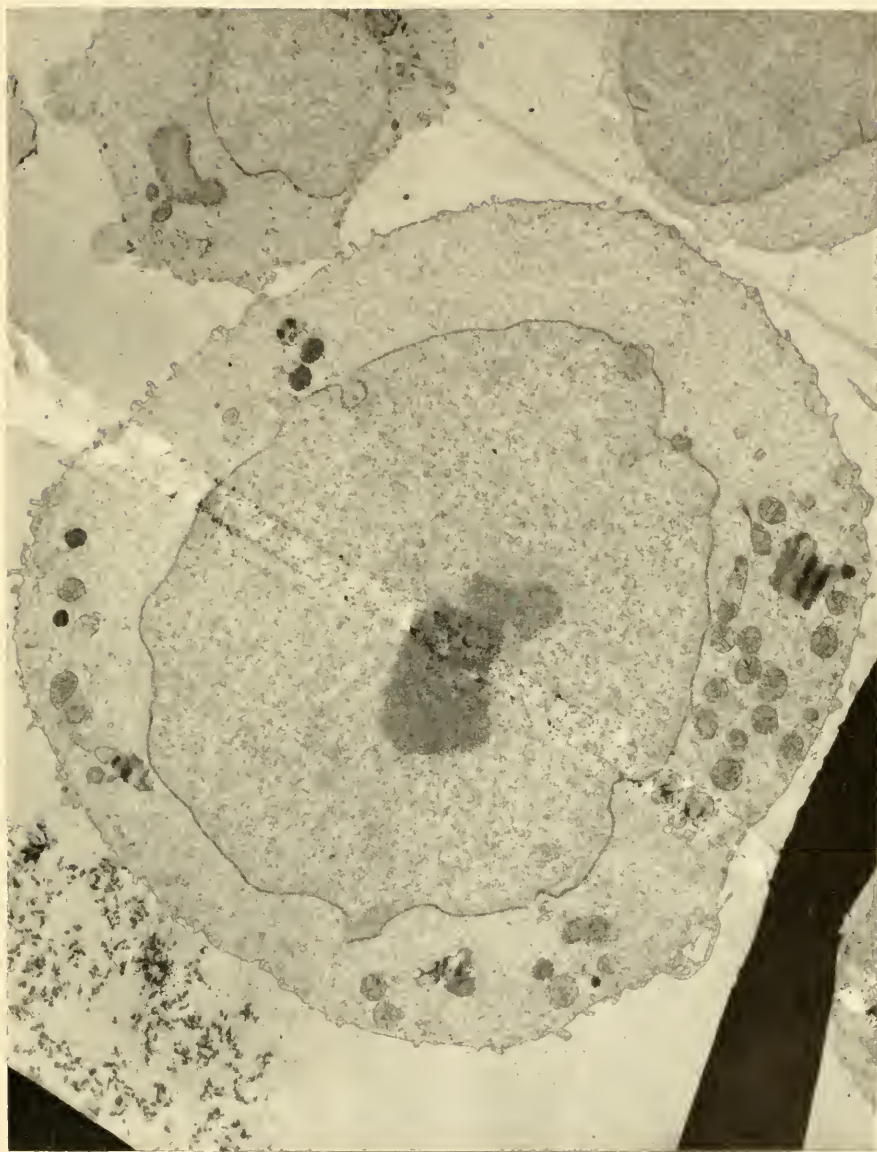


Fig. 3.—Similar cells 42 hr after 300 rads of X-rays. About 10 per cent of the cells turn into giants and an example of these is seen in the centre. The majority of the cells only double in volume (i.e. diameter increased by less than 30 per cent) and two of these are shown at the top.

giants with up to thirty times the normal volume (Fig. 3). Even after cell division has stopped, DNA and protein synthesis proceed normally for some twelve to fifteen hours. Interference with protein and DNA synthesis only becomes apparent in these cultures some 36 hr after irradiation when the cells can be seen microscopically to be degenerating.

Cell death under these conditions has frequently been attributed to chromosome damage and undoubtedly the loss of large amounts of genetic material must render a cell non-viable. But this cannot be the only mechanism, for radiosensitivity and chromosome damage do not go hand-in-hand (Oakberg and Minno, 1960; Bender, 1960; Sharman, 1959). The possibility must be envisaged that the time interval between irradiation and cell death is needed for the metabolic development of the injury as is the case for all radiation effects. That in rapidly dividing cells mitoses occur during this essential time interval need not imply that mitosis itself is a necessary step for the process of mitotic death; the mitosis may be coincidental. Evidence for this view is derived from experiments in which cells are prevented from division after irradiation by a treatment that does not suppress metabolism. If the leukaemia cells are kept after irradiation at 22°C for 18 hr and then returned to their normal temperature of 37°C, cell death takes place without intervening mitosis. At the lower temperatures the cells still metabolize although no division occurs (95 per cent of the unirradiated cells survive 18 hr at 25°C).

In this connection it is worth emphasizing that there is no evidence that allows us to link chromosome abnormalities with radiochemical damage to nucleoprotein. The concept that the ionizing particle severs the chromosome or chromatid thread on passing through it finds no support from *in vitro* studies of DNA (Lett *et al.*, 1961a, b) nor does it explain the biological data (Revell, 1959). A time interval is always necessary between irradiation and the appearance of the chromosome "break". To see a break the cell has to be studied in metaphase or anaphase, yet the irradiation has to be carried out hours earlier while the cell is in the resting stage or in early prophase. The explanation that the interphase chromosomes are more slender structures that can be severed more easily by an ionizing particle than the visible chromosomes of mitosis is invalid since cells irradiated during mitosis show chromosome "breaks" at a high frequency in the next mitosis.

#### RADIOSENSITIVITY, RADIOSENSITIZERS AND INTRACELLULAR PROTECTORS

Many factors are likely to play a part in determining the great



variation in the radiosensitivity of cells, in particular those of micro-organisms. One aspect which we have been investigating is the presence within the cell of some substances, e.g. sulphydryl compounds or bacterial pigments, which can act as protective agents and reduce the magnitude of the initial chemical lesion by one of the energy transfer or repair processes discussed in our other paper in this symposium. We must stress that the presence of intracellular agents can at best be only *one* of the factors making for high radio-resistance. We have tested this hypothesis by comparing the dose needed to damage the same "marker" molecule within different cells having varying radiosensitivities. In these experiments large doses have to be used since a radiochemical reaction unmagnified by metabolism is being studied. The cells are irradiated at 0°C and then immediately broken up in a Hughes press at -25°C. The homogenate is kept frozen until the actual assay occurs. The dose needed to inactivate the enzymes of the Krebs cycle in *Micrococcus sodensis* with an LD<sub>37</sub> of 33,000 rads (220 kV X-rays) was approximately five times that necessary for the much more radio-sensitive *Pseudomonas fluorescens* (LD<sub>37</sub>, 1,800 rads). The protection of the enzymes in the cytoplasm of the micrococcus may be related to the presence of a carotenoid pigment since this is much more sensitive to irradiation within the cell than in isolation. Sulphydryl protection does not appear to be operative since the concentration of SH groups is less in the micrococcus than in the pseudomonas. The DNA of the cells does not appear to be protected since approximately similar doses of radiation (100,000 to 200,000 rads), suffice to reduce the molecular weight to one half (i.e. to produce one "double" break, see p. 14) in pseudomonas, micrococcus, rat thymocytes and mouse leukaemia cells. These measurements were made on DNA extracted immediately after irradiation with X-rays at 10<sup>4</sup>r/min at 0°C. The amount of DNA that could be extracted from the irradiated cells was, however, less than that obtained from non-irradiated controls and this introduces an ambiguity in the interpretation.

Removal of the intracellular protective agents should lead to radiosensitization. Iodoacetate, which has been shown to enhance radiation damage in mice, readily combines with sulphydryl compounds that could be physiological protectors. Leukaemia cells in tissue culture exposed to iodoacetate at a concentration of 10<sup>-5</sup> M for 30 min prior to irradiation (but *not* when given after irradiation) are 30 per cent more sensitive to X-rays than those that have not been treated: 300 r produce an effect for which 375r would otherwise be necessary. To make this experiment easily interpretable the treatment with iodoacetate was sufficiently mild so as not to interfere with the growth of the cells in any

way, and it only blocked 30 per cent of the total SH groups of the cell. Treatments that lead to the loss of more SH groups were toxic. It is not surprising that the extent of sensitization was so small under these conditions. Pre-treatment of bacteria with SH reactors leads to very much greater sensitization. Exposure to  $10^{-4}$  M iodoacetamide doubles the radiosensitivity of *Pseudomonas fluorescens* and it increases that of *Micrococcus sodensis* seven times. Post-irradiation exposure to iodoacetamide is without effect on the survivors. These very great changes in radiosensitivity may not be due solely to the removal of protective SH-compounds but may be due to a change in the physiological state of the cell due to the blocking of SH-enzymes (Alexander and Mikulski, 1961).

IS THERE A MOLECULE OR A SERIES OF MOLECULES WHICH MAY BE FOUND ALTERED IMMEDIATELY AFTER IRRADIATION *IN VIVO* WITH DOSES GIVING IMPORTANT ANATOMICAL LESIONS AFTER A LONG LATENCY?

All organic molecules are susceptible to damage by radiation and in the irradiated cell almost every constituent is liable to be altered chemically by the direct or indirect action of radiation. A consequence of this almost complete absence of selectivity is that a high proportion of the few reactions that occur will be harmless as they do not involve a molecule (or structure) of which nearly every one is essential to the cell.

Two kinds of molecules—enzymes and nucleoproteins—are at first sight possible candidates for the essential lesion.

### *Enzymes*

When purified enzymes are irradiated *in vitro*—with large doses—a decreased activity is regularly observed.† When enzymatic activities are tested very soon after moderate irradiation in homogenates, tissue slices or whole organisms inhibition—even slight inhibition—(cf. DNA and protein synthesis in cells irradiated in tissue culture is rarely noted. On the contrary, anabolic as well as catabolic enzymatic actions are generally found increased, sometimes as much as ten times (for

† The chemical mechanism by which ionizing radiations inactivate enzymes depends on whether the action is direct or indirect. If direct, a single primary ionization is often sufficient to disorganize the whole of the secondary structure of a protein by causing the breakage of hydrogen bonds (Alexander *et al.*, 1959). The radicals from water (i.e. indirect action) react chemically with the protein, but the majority of the reactions occur on groups that are not essential for biological activity and these reactions do not lead to inactivation. Only occasionally (ranging on average from one in ten to one in one hundred) does a radical inactivate by reacting with an essential site. Consequently, many ionizations have to occur in water before a protein is inactivated by “indirect action” and this therefore is much less efficient than direct action.

ref. Bacq and Alexander, 1961). We shall come back to this point later.

Another and perhaps more serious objection to an enzyme theory is that almost all the biological effects and, in particular, cell killing are brought about more effectively by densely ionizing radiations such as  $\alpha$ -particles and fast neutrons than by sparsely ionizing radiations such as hard X-rays or  $\gamma$ -rays. For a radiochemical reaction, shown to occur in the cell, to be a candidate for the role of primary lesion, it must, at least qualitatively, show the same relative efficiency for radiations of different ionizing densities. This requirement effectively eliminates most radiochemical reactions studied so far since in almost every case densely ionizing radiations were shown to be less efficient. This is the case for the inactivation of enzymes where  $\alpha$ -particles are many times less effective than sparsely ionizing radiations.

Gordy and Shields (1958) have claimed that when proteins are irradiated in the dry state, the energy is funnelled into the disulphide bonds and Ehrenberg and Zimmer (1959) have ascribed great biological significance to this reaction. The experimental evidence for this hypothesis was the observation that the electron spin resonance patterns after irradiation of protein and of the sulphur-containing amino acid cystine were similar. We have investigated this claim (Libby *et al.*, 1961) and Fig. 4 shows that the pattern of protein and cystine are quite different and that there can be no question that the same radicals are produced. The patterns only resemble one another

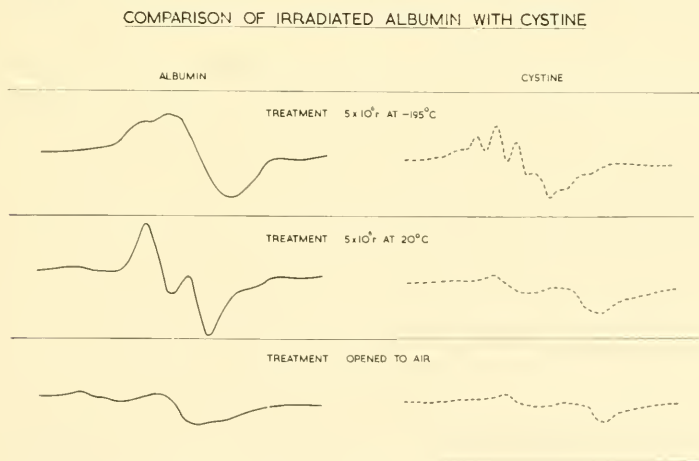


Fig. 4.—Comparison of the electron spin resonance pattern obtained by irradiating bovine serum albumin and cystine with  $^{60}\text{Co}$   $\gamma$ -rays *in vacuo*. The irradiations and measurements were carried out at  $-195^\circ\text{C}$  and at  $20^\circ\text{C}$ . After measurement, the samples were opened to the air and their ESR remeasured (for experimental details see Libby *et al.*, 1961).

when oxygen is admitted and this is due to the fact that the radicals become peroxidized in both substances. Chemical analysis of irradiated protein confirms that cystine is not preferentially destroyed (Alexander and Hamilton, 1960).

### *Deoxyribonucleic Acid (DNA)*

On quantitative grounds, DNA is a more likely candidate for the primary lesion than enzymes, since it is probable that every molecule is unique. It is possible therefore that damage to only a few molecules per cell could affect survival. There are however a number of difficulties with this interpretation (Alexander, 1960; Lett *et al.*, 1961a, b) and before considering these we shall summarize the available information about the chemical and physical changes that DNA undergoes on irradiation.

*Indirect action.* This results in far-reaching chemical changes such as the opening up of the imidazole ring of the purines (Hems, 1960) and the formation of peroxide groups on the pyrimidines (Ekert and Monier, 1959). From the point of view of cell death (though not necessarily for mutations) reactions that alter the macro-molecular properties of DNA (such as main-chain scission or cross-linking) are probably more important since every one of these is liable to destroy the biological integrity of the molecule while isolated chemical changes in one of the bases need not invariably be damaging.

In dilute aqueous solution X-rays reduce the viscosity of DNA (Taylor *et al.*, 1948). This reaction is now known to be the result of rupture of the molecule which occurs whenever a break has been produced in both of the constituent chains within a distance of some five nucleotide units. A detailed investigation by Moroson and Alexander (1960) has shown that for a DNA molecule of  $5 \times 10^6$  molecular weight (number average) 65 OH radicals are necessary before a break becomes apparent. The majority of the OH radicals are used up in producing isolated (non-coincident) breaks that remain hidden.

There has been a great deal of contradictory data concerning the post-irradiation fall in viscosity of DNA following irradiation in dilute solution, and complex mechanisms involving unstable peroxides and phosphate esters have been proposed (see review given in Bacq and Alexander, 1961). Although these reactions may occur to a limited extent, two main causes for this *in vitro* post-effect have been found (Alexander, 1959).

1. DNA is known to be unstable in solutions of low ionic strength. If less than  $10^{-3}$  M of salt is present the molecule denatures on



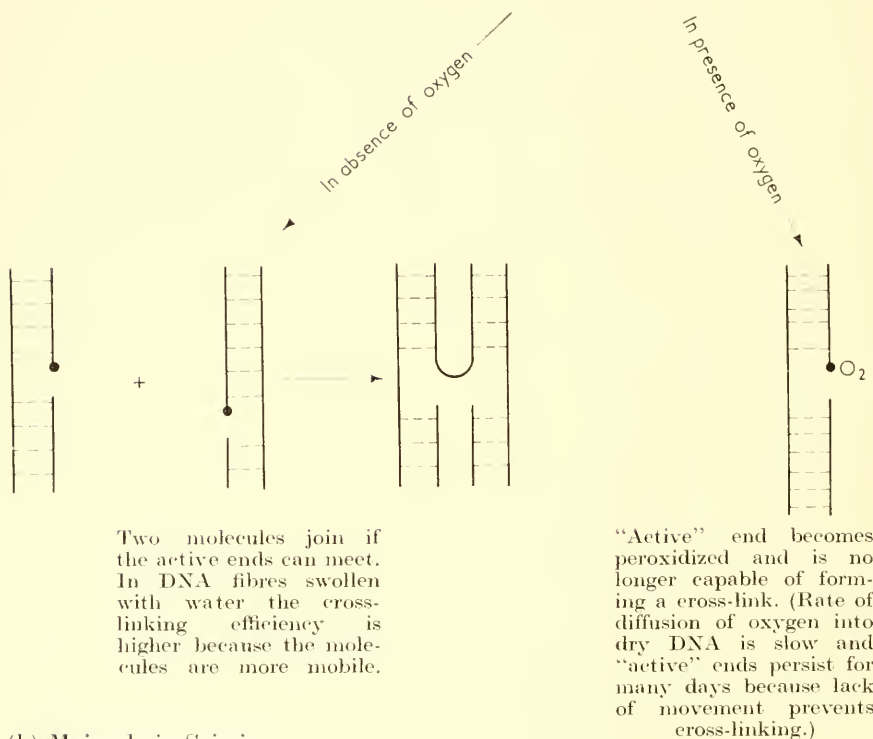
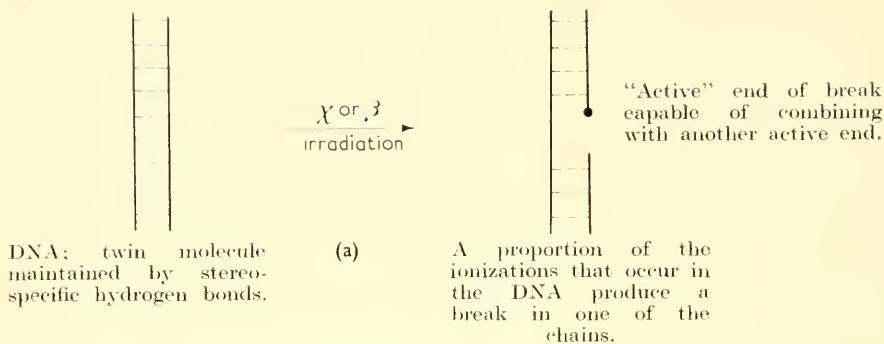
standing and takes up a more coiled configuration. This change is accompanied by a fall in viscosity. This type of denaturation occurs more readily if there are "hidden breaks". Hence if DNA is dissolved at ionic strengths of less than  $10^{-2}$  M the molecule coils up, denatures slowly and this coiling leads to a reduction in viscosity after irradiation. This type of after-effect is completely prevented if DNA is irradiated at ionic strength approaching physiological.

2. DNA is very susceptible to oxidation by dissolved chlorine which produces breaks in the main-chain in the same way as OH radicals do (Moroson and Alexander, 1960). If DNA is irradiated in solutions of sodium chloride, some of the OH radicals react to give dissolved chlorine. This will then react slowly with DNA to produce breaks (Alexander, 1959). The addition of sodium thio-sulphate immediately after reaction prevents this type of post-effect by removing the dissolved chlorine. If steps are taken to prevent these two reactions the fall in viscosity following irradiation is small and represents less than 20 per cent of the total change.

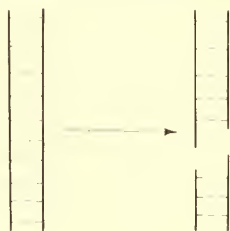
*Direct action by sparsely ionizing radiations.* The changes produced when DNA is irradiated in the dry state or as a concentrated gel are extremely complex and the observed effects depend critically on the experimental conditions (Alexander *et al.*, 1960; Lett *et al.*, 1961a, b).

DNA containing 20 per cent of moisture or less is almost unaffected by irradiation and the presence of oxygen has relatively little effect. DNA fibres containing an equal weight of water are readily degraded if irradiated in the presence of oxygen but become cross-linked when irradiated in its absence. Cross-linking is recognized by an increase in molecular weight and at higher doses the formation of an insoluble gel. We have explained these findings by postulating that the result of an ionization is to produce a break of one of the chains in such a way that one of the ends is active (e.g. a free radical) so that it can combine with another end to produce a cross-link. The active ends combine readily with oxygen after which they are no longer capable of cross-linking. In the dry DNA, diffusion of oxygen is slow but molecular movement is also limited and few of the active ends have an opportunity to form cross-links and the majority are wasted. When the DNA is swollen with water the opportunity for interaction between active ends is greater, but there is now a competing process due to the rapid penetration of oxygen. This complex set of reactions is summarized in Fig. 5.

### (a) Cross-linking



### (b) Main-chain Scission



This occurs when there is a break in each of the adjacent chains less than about 5 nucleotide units apart.

This is produced by radiation:

1. Every time a DNA molecule is traversed by an  $\alpha$ -particle\* (600 eV double break).
2. When a cluster of ionizations (or other high energy event) is formed by sparsely ionizing radiations (850 eV double break).
3. When by chance two isolated breaks come into juxtaposition. From statistical calculation one “double break” will occur for every 70 random single breaks. This mechanism is responsible for main-chain scission by the indirect action of H and OH radicals formed in the water.

\* Some cross-links are produced at the same time as main-chain scission by  $\alpha$ -rays due to the relatively sparsely ionizing  $\delta$ -rays.

Fig. 5.—Changes in the macromolecular properties of DNA brought about by ionizing radiations.

Both cross-linking and degradation have been observed in the DNA in heavily irradiated cells. The DNA in thymocytes and in mouse leukaemia cells suffers only degradation and no evidence for cross-linking, while cross-linking predominates when the sperm of fish are exposed to sparsely ionizing radiations (Alexander and Stacey, 1959).

*Irradiation with  $\alpha$ -rays.* The effect of densely ionizing radiations such as  $\alpha$ -rays from polonium is initially less complex,<sup>†</sup> since only degradation is observed, the efficiency of which is independent of conditions. Quantitatively, a double strand of DNA is severed every time it is traversed by an  $\alpha$ -particle. This corresponds to one "double break" for every 650 eV of energy deposited (Alexander *et al.*, 1961).

*Biological implications.* These studies show that DNA is drastically altered by radiation. Some of the effects produced are qualitatively different in the presence of oxygen, but there is no evidence to suggest that oxygen influences the total number of molecules affected.

$\alpha$ -rays are slightly more effective than sparsely ionizing radiation (650 eV as opposed to 800 eV) in producing main-chain scission (double breaks) and this is therefore a radiochemical reaction in which the relative efficiencies of different radiations are at least qualitatively the same as for biological effects. It is tempting therefore to speculate that double breaks may constitute an important primary lesion. But this hypothesis fails to provide an explanation for the enhancement of radiation of sparsely ionizing radiations by oxygen which does not interfere with main-chain scission, but only prevents cross-linking. Another difficulty is that one would expect cross-linking to be at least as effective as main-chain scission in rendering DNA biologically useless. If damage to DNA were an important primary lesion then the combined effect of cross-linking and main-chain degradation must be considered. On this basis, electrons are more efficient in inactivating DNA molecules than  $\alpha$ -rays. This is confirmed by irradiation of transforming principle for which the inactivation dose increases with increasing LET (Fluke *et al.*, 1952).

#### DAMAGE TO MEMBRANES AND INTRACELLULAR BARRIERS

It seems that on the basis of radiochemical studies, we have to reject the view that damage to DNA or DNA-protein constitutes the primary

<sup>†</sup>  $\alpha$ -rays are always accompanied by less densely ionizing  $\delta$ -rays which comprise some 20 per cent of the total energy deposited by  $\alpha$ -rays. These  $\delta$ -rays give rise, in the absence of oxygen, to cross-linking and this complicates the results obtained with  $\alpha$ -rays.

lesion at least for processes leading to cell death. This is in agreement with the observation that cells without DNA (red cells or reticulocytes) or anucleate fragments of cells (amœbae, *Acetabularia mediterranea*) show typical radio-lesions. Having eliminated enzymes and DNA, the whole concept that a biologically active entity is involved in the primary process may have to be abandoned.

We suggested in 1955 (cf. Bacq and Alexander, 1961) that the increase in enzymatic activity which is so frequently seen in irradiated organisms soon after irradiation† could be explained if radiations broke down internal barriers within the cell—the enzyme release theory.

Not only is there an intracellular increased enzymatic activity but also a leakage of many enzymes (for instance, DNase, peptidase, two transaminases and two dehydrogenases—for ref. Bacq and Alexander, 1961) in the plasma and the urine. Such leakage of enzymes is now well understood as a good but unspecific sign of cellular damage; it can be elicited by anoxia or by drug-poisoning.

Increased enzymatic activity in cell homogenates or tissue slices of whole organisms can be theoretically explained in three ways (see ref. in Bacq and Alexander, 1961).

(i) Increased synthesis of the enzyme; this is the case after irradiation for tryptophane peroxidase in the rat, an adaptative enzyme depending for its regulation on the adrenal cortex.

(ii) Destruction of an inhibitor. Many enzymes (the DNases and RNases) are known to be inhibited by substances that occur naturally in the cell or in circulating fluids. Feinstein and Ballin (1953) believe that this is the case for a cathepsin.

(iii) Increased contact between enzymes and substrates. De Duve (1958) has conclusively demonstrated that certain granules (called lysosomes) contain a series of enzymes (acid DNase, acid RNase, acid phosphatase, cathepsin,  $\beta$ -glucuronidase and aryl-sulphatase) which *in vitro* react poorly or not at all with their specific substrate unless their “membrane” has been damaged by freezing, osmotic imbalance or by mechanical means. One should never forget that the DNase which is in these lysosomes (and also in the mitochondria) has no substrate available on the spot; it must travel a long way (if we think in molecular distance) and pass through many cytoplasmic barriers and through the nuclear membrane before reaching some DNA.

† Recent observations by Libinon (1959) have shown that in the bone-marrow of rabbits the activity of acid and alkaline ribonucleases is increased three to five times about one hour after the beginning of irradiation (1000 r,  $\gamma$ -rays  $^{60}\text{Co}$ , 15 r/min.) The activity of the DNases is increased 50 to 100 per cent one hour after irradiation.



In a certain way our theory is so obvious that it appears to us as a "lapalissade"<sup>†</sup>.

The cell is a network of phospholipid membranes and one of their chief functions is to keep certain substrates and enzymes apart. If these barriers are disrupted, either by forming holes or altering their selective permeability in some way, serious biochemical disturbances will result.

The release of degradative enzymes, for example, could give rise to damage to nucleic acids and proteins. But this is only one of the possible mechanisms by which an interference with internal cell structures could lead to cellular lesions, and reactions such as coagulation of nucleoprotein by calcium ions may have to be considered. Possibly the actual biochemical process that is initiated by membrane damage may vary for different cells and for different types of lesions.

To break down fine intracellular structures, several ionizations acting in conjunction may be necessary and this could explain the superiority of densely ionizing over sparsely ionizing radiations in initiating cellular lesions (i.e. a given amount of explosive is much more effective in knocking down a wall in the form of one cannon shell than in the form of many rifle bullets none of which can penetrate).

A mechanism by which oxygen enhances the radiation lesions of sparsely ionizing radiations can be envisaged for membrane damage. The phospholipid membrane may undergo a chain reaction with oxygen which is initiated by an ionization for they contain unsaturated fats and in this way the effect of a single ionization may be greatly multiplied. With densely ionizing radiations this multiplication effect may not be necessary as the damage produced by several ionizations close together is already sufficient.

The possibility that damage to membranes contributes to the killing of lymphocytes by radiation is indicated by electron microscopic studies. Four hours after 1,000 rads are given to the thymus of rats all the cells and, in particular, the nuclei show gross morphological abnormalities (i.e. pyknosis) when fixed with heavy-metal-containing fixatives and then stained by the methods developed by Trowell (1952). Yet when these same cells are examined in the electron microscope (with osmium fixation), the shape of the nucleus is quite unaltered and no morphological abnormalities can be seen (Alexander, 1961). The heavy metal fixative used to show up pyknosis causes clumping of isolated nucleoprotein whether this has been irradiated or not. We believe that

<sup>†</sup> The early release of enzymes may be the reason for the primary step of their inactivation which may be observed several hours or days after irradiation. Enzymes when linked to mitochondria or microsomes are inactive but protected against destruction; when liberated, they may become a substrate for proteases similarly released.

irradiation makes it possible for the fixative to penetrate into the cells and that the pyknotic changes are not caused by the irradiation, but are due to access of the fixative.

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## DISCUSSION

POWERS: What was the effect of heating in your experiments on paramagnetic resonance? In other words do you admit the possibility of changes in your material due to heating?

ALEXANDER: These experiments were usually carried out at a temperature of  $-195^{\circ}\text{C}$ . Then the sample was warmed up to room temperature. A change in the spectrum resulted. The sample was kept at room temperature for a day, and showed further changes of the spectrum. We did not cool the sample again, since it is quite evident that at room temperature irreversible changes could have occurred as radicals acquired the possibility of meeting and recombining, which does not occur at a temperature of  $-195^{\circ}\text{C}$ .

POWERS: How do you explain these changes?

ALEXANDER: Particularly large changes occur when air is admitted. On heating the changes are less considerable. When irradiation was performed at room temperature different changes of the signals were also provided. I cannot go into the details but would like to emphasize the importance of our results in determining the role of sulphur.

POWERS: I do not think that these data support your surmises on the role of sulphur.

ALEXANDER: When irradiation was carried out in the presence of oxygen, ESR signals of the irradiated cysteine and proteins were similar, but if during irradiation oxygen was lacking, the cysteine and protein signals differed. We believe that the similarity of these signals is due to the oxygen, in the presence of which peroxide radicals are formed.

LEBEDINSKY: What experimental data confirm the very valuable, in my opinion, suggestion about the importance of the membrane permeability disturbances?

ALEXANDER: I hope to demonstrate this in the future by excluding other possible causes, since it would hardly be possible to obtain direct proof. Besides this, it is probably possible to get physiological and biochemical data confirming this viewpoint in an indirect way. But I have no direct evidence except the fact that following irradiation of erythrocytes with several hundred roentgens a leakage of the potassium is observed while at the same time we have shown that there is a change of the surface potential following such a dose. However, this is only a suggestion, not a proof.

BLUMENFELD: What could you say about the form, width and position of the ESR signals which you obtain from protein at liquid nitrogen temperature?

ALEXANDER: We did not obtain ESR signals with unirradiated protein whether this was denatured or native.





# MECHANISMS INVOLVED IN THE INITIATION OF RADIOBIOLOGICAL DAMAGE IN AEROBIC AND ANAEROBIC SYSTEMS

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## SUMMARY

Loss of reproductive integrity in spores, vegetative bacteria and cells of higher plants and animals is in general a result of damage to a very small number of macromolecules which are important for proliferation. In dry spores a good deal is now known about the nature and lifetime of intermediates in the chemical reaction chains leading up to the damage to these macromolecules. Water, when present in small amounts, profoundly modifies the reaction chains and shortens the lifetime of many intermediates. In cells of high water content a considerable fraction of the damage may proceed from the radiolysis of the water. The consequence of a particular kind of macromolecular damage, which has arisen along a particular chemical pathway, may be greatly influenced by nutritional factors, both before and after irradiation. The influence of the chemical and nutritional factors is interrelated.

## THE NATURE OF THE PROBLEM

The aim of radiobiologists is to trace the course of events forward as far as possible, from the initial interaction between the ionizing particles and the molecules present in the living cell, through the formation of excited states, unstable intermediates of finite life, and on to chemical changes which would be stable in a non-living system but which represent functional defects when they occur in certain organelles of the living cell. These functional defects become in turn the starting point for a further sequence of chemical changes, and may be regarded as primary lesions at the biochemical level.

The ionizing particles are moving too fast to excite molecular vibration levels directly. The energy is transferred first to the electronic system of the recipient molecule and, almost always, in amounts which are large compared with the activation energies for the ordinary chemical reactions which the molecules are known to undergo. Thus in the irradiated cell there arise, almost at random, centres of energy absorption of such magnitude that many alternative pathways of chemical reaction become possible simultaneously at each site. As

Platzman and Franek (1958) have pointed out, there also arises simultaneously in a polar medium around the molecules, which are ionized, electric fields of sufficient intensity to break many—perhaps 20—hydrogen bonds in the immediate neighbourhood.

It is clear, therefore, that from the instant of irradiation thousands of different reaction chains are proceeding simultaneously.

Our concern is to associate particular reaction chains with particular forms of radiation response in the cell or in the organism. It is unlikely that any generalization will be found to cover all forms of response, as two examples will suffice to show.

When ionizing radiation provokes the sensation of vision (Lipetz, 1960) the chain is evidently a short one. The response occurs in a small fraction of a second, and the reaction chain is probably confined to those changes initiated in the immediate vicinity of the light-sensitive elements of the retina, or to the direct excitation of these elements. Similarly, changes in the swimming habits of *Daphnia magna*, resulting from exposure to a brief pulse of X-radiation, are traceable to disturbances in the nauplius eye (Baylor and Smith, 1958), which in turn may consist of a radiation-induced reduction of certain pigment molecules, since the effects of the radiation are mimicked by reducing dyes and opposite to those produced by dyes having a redox potential greater than +0.062 (relative to the hydrogen electrode).

The retina probably constitutes an almost ideal radiation detector, since the stimulation of a small number out of a very large array of identical molecules leads to a recognizable biological response.

At the other extreme, the chain of events leading to loss of proliferative capacity in bacteria, in higher plants and in mammalian cells is a long one, and this is the subject of this paper.

#### CONSIDERATION OF DOSE-RESPONSE RELATIONS AS A GUIDE TO THE ANALYSIS OF THE REACTION CHAINS WHICH MAY BE IDENTIFIED BY PHYSICAL AND CHEMICAL MEANS

When allowance is made for experimental error, the analysis of dose-response relations cannot rigorously establish the number of initiating events. Zimmer (1960) has, for example, shown that it is possible to devise a model with a particular distribution of target size among a population of organisms, (mathematically related to the multiplicity of the events needed to inactivate each target), which would lead to a dose-survival curve which is indistinguishable, within the limits of experimental error, from an exponential curve. It is important to recognize this but, as Bertrand Russell has remarked, "It is a peculiar

fact about the genesis and growth of new disciplines that too much rigour too early imposed stifles the imagination and stultifies invention". In my view, the analysis of dose-response relations is still one of the most powerful means at our disposal for limiting our search—among the tens of thousands of reactions which are proceeding simultaneously in the irradiated cell—for those which lie in the pathway to the loss of reproductive integrity. We may remind ourselves that in genetics, the mathematical analysis of segregation frequencies pointed to the physical existence of permanent structures carrying the genes in a linear array before the microscopic study of *Drosophila* salivary gland chromosomes revealed differences in band pattern between mutant forms. On the basis of an analysis of loss of reproductive integrity by vaccinia virus Lea and Salaman (1942) reached the conclusion that the genetic material of the virus must occupy a small fraction of the total volume before a nuclear body was demonstrated by electron microscopy. Similarly, Preer's (1948) analysis of the radiation inactivation curve of kappa in *Paramecium* yielded an estimate of its size which exceeded the limit for visibility in the light microscope, and led to the identification of the kappa particles.

By the criterion of the observed dose-response curves, loss of reproductive integrity would be judged, in almost all cases so far studied, to proceed from energy dissipated in the cell, either by a single particle or by, at most, a few ionizing particles. Dr. E. L. Powers and his collaborators (1957, 1961) have published some extremely well defined linear relations between the log surviving fraction and the dose for spores of *Bacillus megaterium* irradiated in the dry state. A very similar relation for *Serratia marcescens* irradiated in aerobic suspension has been observed recently by my colleague Dr. Dewey (unpublished). After a small curvature near the origin, the experimental points define a straight line. Dewey's observations are remarkable in showing that the strictly linear relation is maintained over almost ten powers of ten. The logical interpretation of the linear part of this curve is that when a population of organisms is exposed to a certain increment of dose, the probability of loss of reproductive integrity is the same for each organism and independent of the dose to which it has previously been exposed. For small increments of dose it is also strictly proportional to the dose increment. In physical terms, loss of reproductive integrity in any given bacillus depends only on the energy delivered to the cell by a very small number of ionizing particles. If the experimental observations had defined a line which passed accurately through the origin, we should be able to infer that the loss of reproductive integrity was strictly independent of the reaction chains initiated by all other particles

than the one which is effective. It would not imply that one ionization (or one excitation) is the initiating event at the physical level, but only that one particle is involved.

From the slope of the log survival curves, we know that the target within which the chemical reaction chains leading to loss of reproductive integrity are initiated must contain a great many molecules. Either the molecules themselves must be uniquely important to cellular proliferation, or the reaction chains to which they give rise must involve the inactivation of uniquely important molecules. Attention is thus focused on molecules of the DNA-RNA-protein class.

A second deduction which can be made from the slope of the log survival curves is less agreeable. It is that, for all organisms as large as or larger than bacteria, the target is a small fraction of the volume of the cell. The total number of ions generated in the cell for each electron transit through the target is therefore large (Table 1). It follows that

TABLE I. *Fast electron transits per cell at a dose which gives 37 per cent survivors*

	Radius of cell (microns)	Aerobic $D_{37}$ (rads)	Number of elec- tron transits (LET = 1.7ekV/ $\mu$ ) through the cell	Total number of ion clusters formed
Dry spores	1	$2.5 \cdot 10^4$	3000	$7 \cdot 10^4$
Vegetative <i>E. coli</i>	1	$5 \cdot 10^3$	600	$10^4$
Plant or mammalian cells	10	100	1500	$10^5$

when physical methods, such as electron spin resonance spectroscopy, are used to detect free radical intermediates, the chances that the signal is characteristic of the actual radicals which initiate loss of reproductive integrity is correspondingly small.

#### BIOLOGICAL SYSTEMS IRRADIATED IN THE DRY STATE

##### *Loss of reproductive integrity by spores irradiated in the dry state*

I should like to take the exceedingly beautiful work with dry spores of *B. megaterium* by Dr. E. L. Powers, and his collaborators (this Symposium) as a starting point for my remarks concerning loss of reproductive integrity in aerobic and anaerobic bacteria, and the proliferating cells of higher plants and animals, with which I am personally more familiar. Davis and Hutchinson (1952), working with the closely



related *B. subtilis*, inferred from their studies with very slow electrons that the radiobiologically important material comprises only part of the spore, being surrounded by a shell of very insensitive material 230 Å thick.

The log survival curves observed by Powers are, under all conditions of irradiation, strictly linear over almost their entire length, but show a small curvature towards the origin. The extrapolation number (as well as the slope) varies with the conditions of the irradiation, but is always less than 2. Loss of reproductive integrity in the spore has, therefore, the general features that I mentioned earlier.

Certain features of Powers' work which are relevant to the present discussion may be summarized as follows:

There are four recognizable classes of events which may lead to loss of reproductive integrity. These are characterized by the chemical reactivity, thermal stability and lifetime of intermediates in the reaction chains. Two involve molecular oxygen and two do not.

*Classes of event leading to reaction chains in which the participation of molecular oxygen is not essential*

*Class A* events lead to reaction chains which are equally toxic under all conditions so far tested.

*Class B* events lead to reaction chains which are equally toxic under all conditions tested, except treatment with  $\text{H}_2\text{S}$ . They react with  $\text{H}_2\text{S}$ , the simplest of the sulphydryl compounds, if present during irradiation, to give a product which is non-toxic. The lifetime of these species is such that reaction with  $\text{H}_2\text{S}$  is no longer possible after the end of an irradiation of a few minutes' duration.

*Classes of event leading to reaction chains in which the participation of molecular oxygen is essential*

*Class C* events generate intermediates that react readily with oxygen to give products leading to loss of reproductive integrity. In the absence of oxygen the intermediates in question have a comparatively long life, at room temperature, and may be stabilized against subsequent reaction with oxygen by thermal treatment, or exposure to NO or  $\text{H}_2\text{S}$  after the end of irradiation. The stabilization is less complete if nitric oxide is present during irradiation (see p. 34).

*Class D* events generate intermediates which only become toxic by reaction with oxygen. They have, however, a much shorter lifetime than the intermediates derived from *Class C* events, and are only influenced by oxygen and  $\text{H}_2\text{S}$  if these gases are present during irradiation.

The physical reality of intermediates having lifetimes and chemical

properties closely resembling those inferred from the biological observations, have been most beautifully demonstrated by Dr. Powers and his collaborators by means of electron spin resonance (ESR) spectroscopy.

Further research may well lead to a further subdivision of these classes. As they stand, we may assign relative probabilities to the recognized classes of initiating event as: A-0.24, B-0.14, C-0.38, and D-0.24. Relative probabilities of reaction chains in which the participation of oxygen is essential to those to which it is not, are thus

$$\frac{C+D}{A+B} = \frac{0.62}{0.38} = 1.63$$

The sensitivity of the spores irradiated in oxygen to that when participation of oxygen is excluded, both during and after irradiation, viz:

$\frac{1}{A+B} = 2.63$ , a figure close to the values usually observed for the relative sensitivities of plant and animal cells irradiated aerobically and anaerobically.

## BIOLOGICAL SYSTEMS OF INTERMEDIATE WATER CONTENT

### *The radiosensitivity of seeds, with special reference to water content*

The influence of varying amounts of water on the sensitivity of cells has been studied in seeds (see reviews by Ehrenberg 1955, Caldecott, 1960, Nilan *et al.* 1960, Konzak *et al.*, 1960, Sheldon Wolff, 1960, and Davidson, 1960). In evaluating this work it must be borne in mind that the biological damage induced in the seeds (as also that in spores) has always been examined after hydration and germination.

When seeds are irradiated dry, intermediates of short and long life-time are produced which have much in common with those that lead to loss of reproductive integrity in irradiated spores. H<sub>2</sub>S and nitric oxide present during irradiation decrease the extent of the injury. This is largely due to the influence of these treatments on the course of those reactions which involve participation of oxygen. By ESR spectroscopy it has been established that long-lived radicals induced by the irradiation of dry *Agrostis* seed disappeared fairly rapidly in the presence of nitric oxide (Sparrman *et al.*, 1959).

If irradiated seeds are hydrated in water which is free from dissolved oxygen, the long-lived intermediates which are present at the end of irradiation are stabilized against subsequent reaction with oxygen, or with any other molecule so far tested. The level of damage is approximately



the same as if water ( $\sim 10$  per cent in the embryo) had been present during irradiation.

If, however, the water used for hydration contains dissolved oxygen (at or below  $300 \mu\text{M/l}$ ), greater damage is observed. Evidently, if the oxygen and the water enter the embryo simultaneously, the oxygen competes successfully for long lived radicals, as it does in all cells of normal (high) water content.

It was observed by Caldecott that after thermal annealing for 15 min at  $85^\circ\text{C}$  the long-lived intermediates induced by the irradiation of dry seed are no longer responsive to the presence or absence of oxygen in the water in which the seed is soaked. In this respect also the seed closely resembles dry spores, since it was found by Powers that thermal annealing eliminated the post-irradiation effect of oxygen.

In this connection the interesting observation has been made by

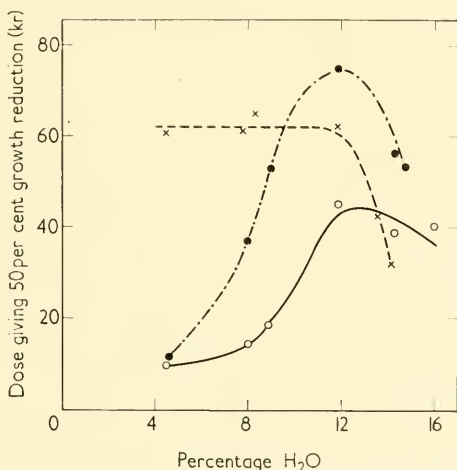


Fig. 1.—Influence of oxygen, nitric oxide and humidity on radiation damage to seeds. (Redrawn, by permission, from Sparrman *et al.*, 1959).

Caldecott (1960) that, whereas the yield of interchanges observed at meiosis in barley plants grown from irradiated dry seed increased linearly with dose when the seeds were irradiated and soaked anoxically:

$$\text{Exchanges} = 1.94D$$

Seeds which were irradiated and soaked in aerated water gave a yield of exchanges which contained an additional term in  $(\text{Dose})^2$ :

$$\text{Exchanges} = 1.76D + 0.31D^2$$

The term in  $D^2$  is more important than that in  $D$  at doses above 6 krad.

The rather close parallel between the influence of small amounts of water on the sensitivity of seed, and on the changes induced by radiation in non-living systems, as illustrated, for example, by the experiments of Clegg (1957) with cellulose fibres, supports the view that we are here concerned with a profound modification in the reaction chains brought about by the presence of water.

When seeds of still higher water content ( $\sim 15$  per cent water in the embryo) are irradiated in the presence of nitric oxide, the radiation damage is enhanced to about the same extent as when the same seeds are irradiated in the presence of oxygen, i.e. the seeds now respond in a manner typical of vegetative bacteria and the cells of higher plants and animals.

The influence of progressively increasing water content on the radio-sensitivity of *Agrostis* seeds, irradiated in the presence of nitrogen, oxygen and nitric oxide, is nicely illustrated by Fig. 1 taken from the paper by Sparman *et al.* (1959).

#### BIOLOGICAL SYSTEMS OF NORMAL (HIGH) WATER CONTENT

Lifetimes of some of the radicals produced by irradiation of seeds having low and intermediate water content are long enough for study by means of conventional ESR spectroscopy. When we attempt to obtain information about the lifetimes of radicals produced in dilute aqueous solutions, or in cells of high water content, a number of difficulties arise, both in experimentation and in interpretation.

##### *Difficulties of experimentation*

Major difficulties arise on account of the short lifetime of the intermediates. We know from the observations of Howard-Flanders and Moore (1958) that essentially all the intermediates involved in the aerobic chains leading to loss of reproductive integrity in *Shigella flexneri* have lifetimes less than 20 msec, and estimates based on the way in which the sensitivity of these cells varies with the concentration of oxygen in the surrounding medium suggest that the lifetimes of the intermediates which react with oxygen may be in the 100  $\mu$ sec range (Howard-Flanders, 1958).

In our laboratory we are attempting to investigate these lifetimes directly, both in chemical systems and in living cells, by means of a pulsed radiation source and pulsed analytical techniques. The source (Boag and Miller, 1959) delivers 1.5 MeV electrons in pulses of 2  $\mu$ sec duration. The dose, which can be delivered in a single pulse, depends on

the volume of material to be irradiated. Suspensions of bacteria have been exposed to 30 krad (Dewey and Boag, 1959, 1960) and volumes of solution suitable for spectroscopic analysis have been exposed to over 100 krad in a single pulse (J. W. Boag and R. E. Steele, unpublished). For spectroscopic analysis a light-source has been developed (Boag, 1957), the output from which during a flash of a few micro-seconds' duration, enables absorption spectra to be recorded at medium dispersion. Such records show the reagents present at a given time after a  $2\text{ }\mu\text{sec}$  exposure to ionizing radiation. By means of a photomultiplier, set to record at the absorption peak of a known reagent, the rate of appearance or disappearance of that reagent may be recorded as illustrated in Figs. 2 and 3.

These figures show certain aspects of the oxidation of a  $10^{-3}\text{M}$   $\text{FeSO}_4$  solution in the presence of  $0.8\text{N}$   $\text{H}_2\text{SO}_4$  and in the presence or absence of  $\text{NaCl}$ . It will be seen that the absorption band at  $305\text{ m}\mu$  characteristic of the ferric ion makes its appearance 20 times more slowly than the absorption (as yet unidentified) at  $240\text{ m}\mu$ . The values of the times required for half the final optical density to appear are  $1200\text{ }\mu\text{sec}$  and  $60\text{ }\mu\text{sec}$  respectively for the two wavelengths. It is evidently quite feasible to carry out kinetic studies for species which occur in the visible and u.v. for lifetimes of this order, or even considerably smaller, with this type of equipment.

The application of similar methods to cell suspensions is, of course, very much more difficult on account of non-specific light scattering, but the methods developed by Britton Chance (1952) and others are, in principle, applicable. ESR spectroscopy is likely to prove more widely applicable than light spectroscopy to the study of radical kinetics in cells which have been exposed to ionizing radiation. In order to take advantage of the high dose-rate during the pulse, Dr. Boag is constructing in our Laboratory a spectrometer in which the magnet has an axial hole through which the electrons may enter the resonant cavity. It is hoped that this will make radicals of lifetimes greater than  $100\text{ }\mu\text{sec}$  open to investigation.

In general, the relatively slow process of diffusion across the cell envelope limits the speed with which the chemical composition of the intracellular fluid may be changed. In certain respects, oxygen is exceptional, since it is consumed in radiochemical reactions (cf. next section).

#### *Loss of reproductive integrity by the bacillus Serratia marcescens*

Several aspects of the loss of reproductive integrity by *Serratia marcescens* have been studied by my colleague, Dr. D. L. Dewey. In all the

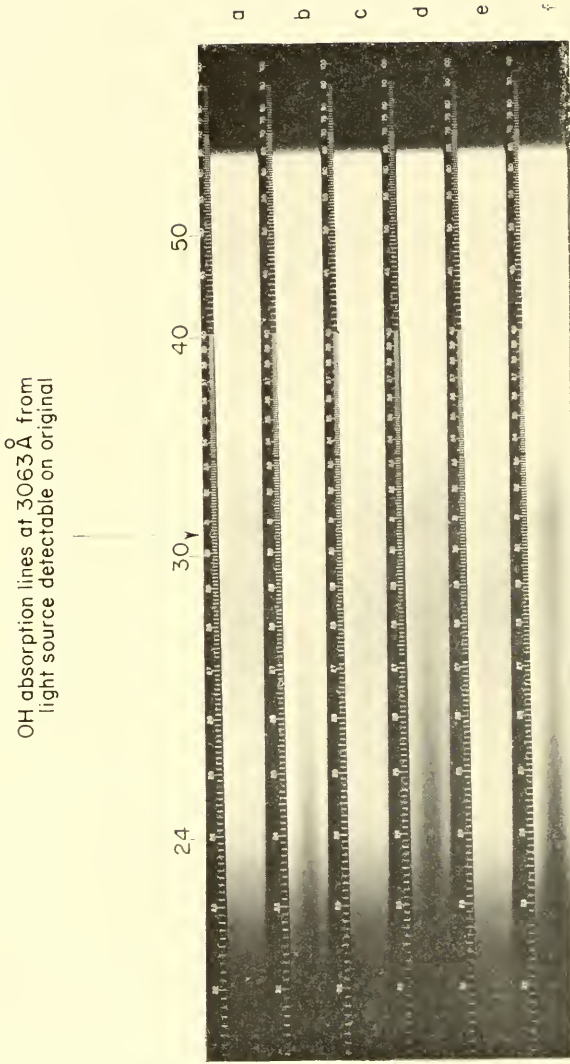


Fig. 2.—Development of u.v. absorption spectrum of  $\text{FeSO}_4$  after irradiation. a, before irradiation; b,  $10\mu\text{sec}$  after irradiation; c, before irradiation; d,  $1\text{ msec}$  after irradiation; e, before irradiation; f,  $90\text{ msec}$  after irradiation. (Reproduced by permission of Dr. J. W. Boag).

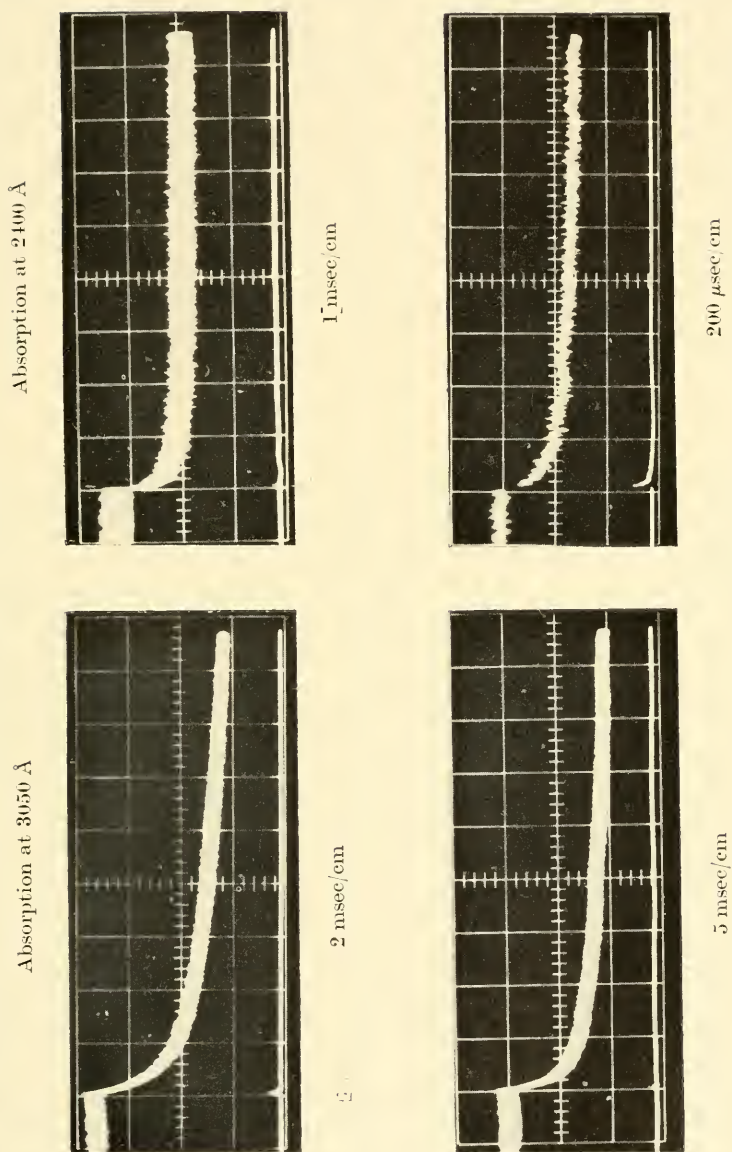


Fig. 3.—Growth of u.v. absorption in irradiated  $10^{-3}$  M  $\text{FeSO}_4$  in the presence of  $0.8$  N  $\text{H}_2\text{SO}_4$  (Reproduced by permission of Dr. J. W. Boag).



experimental conditions which have been investigated so far, the log survival curve is linearly related to dose, except near the origin, where a slight curvature is generally observed when the cells to be irradiated are suspended in a phosphate buffer. In the case of aerobic irradiation at room temperature, the log surviving fraction is linearly related to dose over almost ten powers of ten.  $D_0 = 1.7$  krad, and the extrapolation number is 1.4. These vegetative bacteria are thus  $25/1.7 (= 15)$  times as sensitive as spores of *B. megaterium* irradiated in the dry state under conditions that permit the maximum participation of oxygen. When oxygen is rigidly excluded at the time of irradiation, the sensitivity of *Serratia marcescens* is reduced by a factor of 4. The sensitivity rises, however, very rapidly with increasing  $O_2$  concentration. Assuming the equation proposed by Howard-Flanders and Alper (1957) to be applicable, viz:

$$\frac{S}{S_N} = 1 = (m-1) \frac{O_2}{O_2 + K} \quad (1)$$

where  $S$  is the sensitivity, measured by the slope of the log survival curve, at a concentration of oxygen in the medium equal to  $O_2 \mu\text{moles/l}$ , and  $S_N$  the sensitivity when oxygen is rigidly excluded, then  $m = 4$  and  $K = 4 \mu\text{moles/l}$ . Both constants are, to a first approximation, independent of temperature over the range 0 to  $30^\circ\text{C}$ . This very marked influence of oxygen on radiosensitivity is almost universally observed with proliferating cells, and the question naturally arises as to whether this influence is mediated through the cytochrome system. A number of circumstances make this improbable. For example, Moustacchi (1958) showed that the sensitivity of several cytochrome-deficient mutants of yeast showed the same oxygen dependence as wild type organisms. In the case of *Serratia marcescens*, an involvement of the cytochrome system can be definitely excluded by the observation of Dewey and Longmuir (unpublished) that the oxygen concentrations at which respiration begins to decline at room temperature is very much lower than  $4 \mu\text{moles/l}$ . At temperatures near  $0^\circ\text{C}$  it is less than  $0.1 \mu \text{mole/l}$ .

Sensitivity varies by nearly a factor of 4 over a range of oxygen concentration within which the respiration of the cells is virtually independent of oxygen concentration.

The fact that  $Q_{O_2}$  is maintained approximately constant to much lower oxygen concentrations than  $4 \mu\text{moles/l}$  also shows that the intracellular concentration of oxygen cannot differ from that of the surrounding medium by more than a small fraction of the value of  $K$ . We may, therefore, safely conclude that, unless the nuclear membrane offers substantial impedance to the movement of oxygen, equation (1) is an



expression of the effectiveness with which oxygen can compete at different intracellular concentrations for reaction with radicals or other intermediates to give rise to loss of reproductive integrity.

Measurements between 0 and 30°C show no systematic variation of  $K$  with temperature and enables us to assign an upper limit of 6 kcal/mole to the differences between the activation energies of the two reactions represented by equation (1).

If the lifetime of the intermediate in question is longer than the rate constant for diffusion into the cell (estimated at  $\sim 1$  msec) it may be possible to determine the lifetime directly by the use of our pulsed source. Figure 4 shows the result of an experiment conducted by Dewey

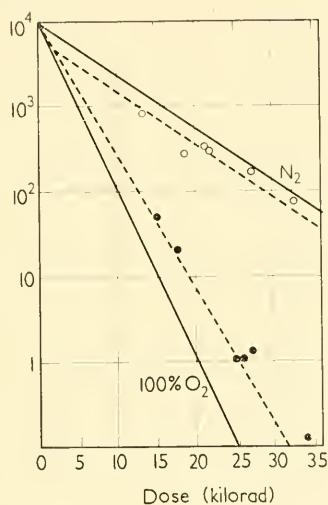
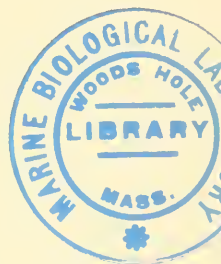


Fig. 4.—Comparison between pulsed and low dose-rate irradiation of *Serratia marcescens*. (Redrawn, by permission, from Dewey and Boag, 1960).

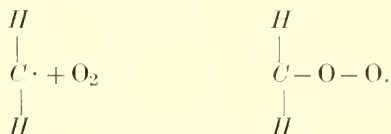
O, 2  $\mu$ sec. pulse;  $\bullet$ , 1,000 rad/min for 0.5 per cent oxygen in the gas phase

and Boag (1959, 1960) in which the effects of doses of radiation delivered at normal dose-rate, and in a single 2 sec pulse, were compared with respect to loss of reproductive integrity. The bacteria were in suspension in a medium maintained at an oxygen concentration of about 10  $\mu$ moles/l by bubbling. Under these conditions the bacteria displayed almost their maximum sensitivity when irradiated at normal dose-rates, but appeared progressively more and more radio-resistant with increasing doses above 5 krad when this is delivered in a single pulse. This was interpreted as due to the radiochemical utilization of all the oxygen initially present in the cells by the reactions arising from the



first 2 to 3 krad of the total dose. Thereafter the cell reacted as an anoxic system.

One of the most striking differences between damage induced in cells of high water content and low water content (dry spores and dry seeds) when each is irradiated in the absence of oxygen, is that nitric oxide enhances the radiation damage in the former, and depresses it in the latter. In dry spores of *B. megaterium* Powers and his co-workers observed less depression when the nitric oxide was present during irradiation than when it was added after irradiation and from this concluded that the nitric oxide slightly enhanced the toxicity of some intermediates while greatly depressing that of the others. The enhancement was first observed by Howard-Flanders in *Shigella flexneri* (1957). As regards the magnitude of its influence on sensitivity at any given concentration, Howard-Flanders and Jockey (1960) find the molecule of nitric oxide to be essentially equivalent to a molecule of oxygen. After the discovery of this phenomenon by Howard-Flanders, a similar effect of nitric oxide on the sensitivity of *Vicia faba* roots was observed by Kihlman (1958); on the sensitivity of tumour cells by Gray *et al.* (1958) and in human liver cells by Dewey (1960a); (see p. 38). In *Serratia marcescens* and *Proteus vulgaris*, Dewey (unpublished) has observed that nitric oxide substantially enhances the sensitivity of anoxic cells, but falls a little short of oxygen in its effectiveness. In each case cells which are exposed to nitric oxide after the end of an anoxic irradiation, show only the normal anoxic sensitivity. Nitric oxide, having one unpaired electron in an outer orbital, readily combines with free radicals to form stable compounds. In this respect it differs from oxygen, which has certain of the characteristics of a bi-radical, so that the product of a reaction between oxygen and a free radical is itself a free radical.



By virtue of its paramagnetism, nitric oxide, like oxygen, readily catalyses transitions in either direction between singlet and triplet states (Porter and Windsor, 1958). Nitric oxide is also a very effective substitute for oxygen in reactions leading to the production of chromosome structural damage by visible light absorbed in *Vicia faba* meristem cells which had previously been exposed to acridine orange (Kihlman, 1959).

Since nitric oxide is known to combine rather readily with certain

haem pigments, including cytochrome oxidase (Keilin, 1955), the possibility of a biochemical role cannot be ignored, especially since post-irradiation respiration sometimes has an important influence on radiosensitivity (p. 41). In all the experiments with vegetative bacteria, and with normal and malignant mammalian cells referred to in this section, this possibility was taken into account by control experiments in which the nitric oxide was added after irradiation.

One further investigation with *Serratia marcescens*, carried out by Dewey (1960b), may be mentioned as possibly indicating the extent to which the radiolysis of water contributes to loss of reproductive integrity in this organism.

A number of years ago Burnett *et al.* (1951) studied the influence of high concentrations of alcohol on the radiosensitivity of *E. coli* B/r, and these investigations were later extended by Marcovich (1958). In repeating the experiments under controlled oxygen tensions Dewey (1960b) finds:

1. A progressive increase in the 37 per cent inactivation dose  $D$  (i.e. a progressive decrease in sensitivity) with increasing glycerine concentrations up to 2M, which conforms to the relation

$$\frac{D}{D_0} = 1 + (\mu - 1) \frac{G}{G + K}$$

where  $D_0$  is the inactivation dose in the absence of glycerine.

2. That when the bacteria are aerobic at the time of irradiation the influence of a given concentration of glycerine is completely independent of (a) oxygen concentration in the range 14 to 1400  $\mu$ moles/l. and (b) temperature over the range 20 to 37°C (Fig. 5).

Under all these conditions  $\mu = 5.25$  and  $K = 0.9$  mole/l.

3. Increasing concentrations of glycerine also progressively lower the sensitivity of bacteria irradiated under strictly anoxic conditions. A two-fold depression of sensitivity below the normally anoxic level has been observed, and extrapolation to infinite glycerine concentration indicates a maximum depression of 2.66. The constant  $K$  was estimated to be 0.8 mole/l. and does not differ significantly from that for aerobic irradiations. Both constants are independent of temperature over the range 20 to 37°C.
4. The influence of glycerine is essentially the same for cells which are irradiated in the presence of nitric oxide as for cells which are irradiated in the presence of oxygen.
5. Equal molarities of ethyl alcohol, glycol and glycerine, which have respectively 1, 2 and 3 OH groups per molecule, are of comparable, though not exactly equal, efficiency.

The fact that a given concentration of glycerine is equally effective in the presence of  $1400\text{ }\mu\text{moles/l}$  oxygen and in the presence of  $14\text{ }\mu\text{moles/l}$  oxygen, which is only a little higher than that necessary to

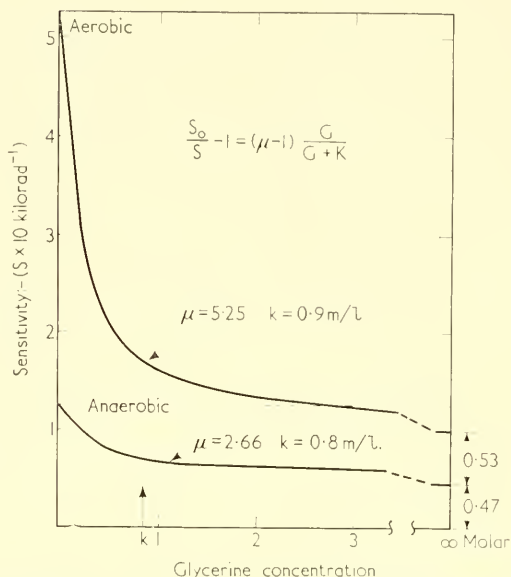


FIG. 5.—Effect of glycerine on aerobic and anaerobic sensitivity of *Serratia marcescens*. (Dewey, 1960 a, b)

bring the bacteria to their maximum level of sensitivity as far as oxygen is concerned, shows that the mechanism involved is not one in which glycerine and oxygen are competing for a radiation-induced radical. It is fairly clear that the action of the glycerine is antecedent to that at which oxygen enters into the reaction chain.

One possibility which is at present under investigation is that, at the molarities in question, the alcohol molecules compete with biologically important molecules for radicals which result from the radiolysis of water. If this hypothesis should prove to be correct, then it would follow from the data presented in Fig. 5 that  $0.47/1.25 (= 38)$  per cent of the anoxic sensitivity of *Serratia marcescens* is due to energy deposited in the biological molecules themselves and 62 per cent to the radiolysis of water. For cells irradiated anaerobically the corresponding figures would be  $0.53/4 (= 14)$  per cent for energy deposited in biological molecules and 86 per cent for the contribution from the radiolysis of water.

*Loss of reproductive integrity by mammalian cells*

It is now clear that the radiation-induced loss of reproductive integrity by mammalian cells has much in common with the same phenomenon in micro-organisms.

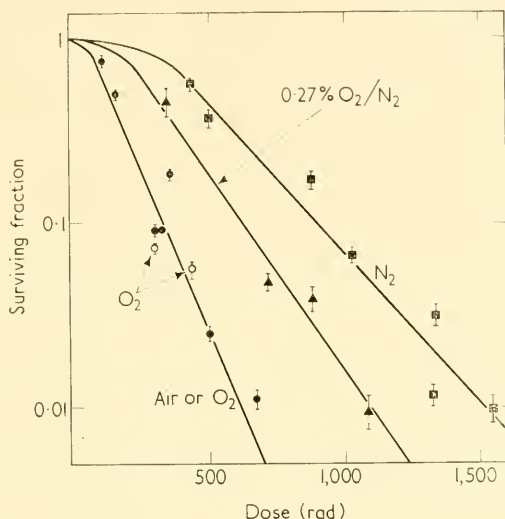


Fig. 6.—Colony-forming ability of human liver cells after exposure to X-rays. (Dewey, 1960 a)

1. Without exception so far, the reported log survival curves are linear for all except the lowest doses and have small extrapolation numbers—mostly  $\sim 2$  and occasionally  $4 \sim 6$  have been observed. Figure 6 presents the data of Dewey (1960a) for human liver cells cultured and assayed by the technique developed by Puck, and exposed to X-rays under various experimental conditions. Figure 7 presents the data of Hewitt and Wilson (1959) for mouse leukaemia cells grown and exposed to  $^{60}\text{Co}$   $\gamma$ -rays *in vivo* and tested for the ability of the irradiated cells to regenerate a tumour.

The shapes of all the curves in Figs. 6 and 7 are remarkably similar, and for cells irradiated at the same oxygen tension the difference between the slopes of the curves for human liver cells and mouse leukaemia cells is close to that expected on account of the different LET of the radiations to which the cells had been exposed.

2. The ratio of aerobic to anaerobic sensitivity for both types of cell is  $\sim 2.5$ . In the case of the human liver cells a sensitivity



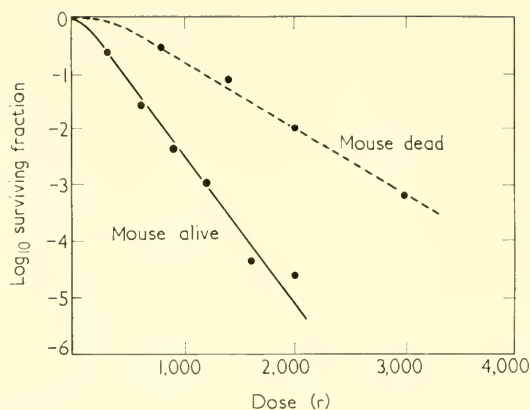


Fig. 7.—Tumour forming ability of mouse leukaemia cells after exposure to  $^{60}\text{Co}$   $\gamma$ -rays. (Hewitt and Wilson, 1959)

approximately midway between the aerobic and anaerobic levels is observed when cells are in equilibrium with a gas phase containing 0.25 per cent oxygen (dissolved oxygen =  $3.5 \mu\text{moles/l}$ ). Nitric oxide enhances the sensitivity of the human liver cells (Dewey, 1960a) as also that of Ehrlich mouse ascites tumour cells (Gray *et al.* 1958) to about the same extent as oxygen.

3. The relation between sensitivity and the concentration of oxygen in the immediate vicinity of the Ehrlich tumour cells at the time of irradiation is shown in Fig. 8. Froese (personal communication), while working in our laboratory, investigated the dependence of the respiratory activity of these cells on oxygen tension. He observed that respiration was maintained at nearly maximum rate down to concentrations  $1 \mu\text{mole/l}$  of oxygen in the liquid phase at which radiosensitivity is not much greater than that observed in the complete absence of oxygen. After making full allowance for experimental error in both sets of data it is evident that, as in the case of bacteria, the variation in the sensitivity of the cells with oxygen concentration cannot be ascribed to an influence of the enzymes of the cytochrome system.

The tumour cells change their sensitivity in less than 1 sec of transfer from one medium to another of different oxygen tension (Deschner and Gray, unpublished).

In mammalian cells, therefore, as in bacteria, we may confidently infer that one, two or, at most, a few ionizing particles initiate loss of reproductive integrity, and that the influence of oxygen concentration during an irradiation of short duration



reflects the chemical properties and lifetimes of intermediates in the reaction chains.

On account of the technical difficulties referred to earlier, no lifetimes have yet been measured directly, but we may infer on

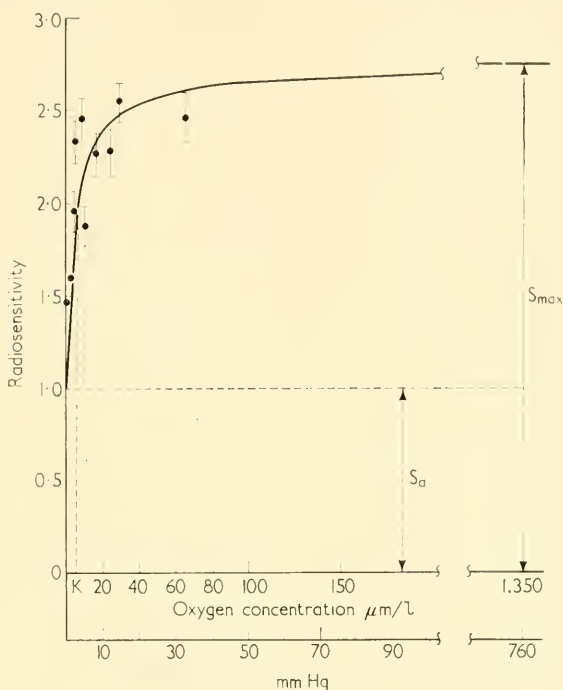


Fig. 8.—Influence of oxygen on radiosensitivity.

kinetic grounds (Howard-Flanders and Moore, 1958) that since the influence of oxygen on sensitivity is represented by equation (1) with  $K \sim 5 \mu\text{moles/l}$  (Deschner and Gray, 1959; Dewey, 1960a), the species with which oxygen reacts probably have lifetimes of the order of 100  $\mu\text{sec}$  when no oxygen is present.

Although the sensitivity of a cell is apparently unrelated to its respiratory activity at the time of irradiation, the sensitivity of plant cells which have undergone a long period of anaerobiosis (Beatty *et al.*, 1956) or which are anaerobic for periods as short as 15 min after the end of a brief irradiation sometimes have a different sensitivity from that of cells cultured aerobically throughout. It is now clear that in this case respiration is important as a source of energy for specific kinds of metabolism, which will be briefly considered in the next paragraph.

*The influence of metabolism on the aerobic and anaerobic sensitivities of cells, with special reference to loss of reproductive integrity*

Witkin (1956) showed that the yield of mutations in certain auxotrophic bacteria, resulting from exposure to u.v. light, is fixed within the first hour after irradiation at a high or low level by the nutrition of the organisms. Low levels appear to be associated with the inhibition of protein synthesis, and in an extreme case no mutation was observed at all, although the loss of reproductive integrity of the cells was little, if at all, affected.

Although loss of reproductive integrity in cells exposed either to u.v. light or to X-radiation has not been entirely prevented by any nutritional variants yet tested very marked changes in the slope, and sometimes also in the extrapolation number, of log survival curves have been obtained both by the use of chloramphenicol (Gillies and Alper, 1959) and by cultivation at reduced temperature (Stapleton *et al.*, 1953) after irradiation. The shape of the log survival curve may also be greatly modified by control of the pH of the medium in which the cells were cultured before irradiation (Hollaender *et al.*, 1951; Stapleton and Adler, personal communication) and in other ways. As a general rule (Alper, 1961), maximum loss of reproductive integrity occurs if cells are caused to grow fast immediately after irradiation by the use of optimal media and optimal temperatures: conversely, more cells survive when growth is slowed or protein synthesis depressed after irradiation. The yield of interchanges when plant cells are exposed to spaced doses of radiation is increased by the inhibition of respiration or chloramphenicol treatment between the two irradiations (Wolff, 1959). Nutritional control for a period of about an hour before an anoxic irradiation may change the yield of interchanges produced in plant cells by a factor of seven (Beatty and Beatty, 1960): This is equivalent to changing the dose by a factor of about 3 since interchanges vary roughly as the square of the dose. Anaerobic metabolism was found to increase the yield, and aerobic metabolism, or the addition of ATP to cells which had been growing anaerobically, to depress the yield.

The interpretation of these observations is in the province of radiation biochemistry, and beyond the scope of this paper. If it be granted that the nutritional control of radiation damage in some way concerns the replacement or repair by normal metabolic processes, of key molecules in the DNA-RNA-protein class, which have been damaged through ionization, excitation and radical formation, then the question may be asked whether or not the extent to which damage may be repaired is the same for all chemical pathways by which the damage was produced. Alternatively, we may ask if damage which is produced by

different chemical pathways is equally reparable by a given form of metabolic control. There is now abundant evidence to show that it is not. An extreme example comes from recent observations of Laser (1960) that starvation of *Pseudomonas* for 48 hr after irradiation completely eliminates damage which is oxygen dependent, but leaves unchanged the damage which does not involve the participation of oxygen in the chemical chain.

Alper and Gillies (1958; 1960a, b) have recently made a special study of this problem using loss of reproductive integrity in *E. coli* B, irradiated aerobically and anaerobically, as test material for the influence of post-irradiation nutritional factors. Alper (1961) finds that the ratio of the sensitivity of organisms irradiated in oxygen and in nitrogen (i.e. the constant  $m$  of equation (1)) is approximately linearly related to the value of  $D_0$  observed for organisms that have been irradiated anoxically, irrespective of the culture condition used to control  $D_0$ . Alper estimates that whereas  $m = 3.6$  under conditions that give maximum injury (no "restoration")  $m = 1.5$  under conditions that give minimum injury (full "restoration").

In other organisms  $m$  may be unaffected by the conditions of culture. The more usual case, however, appears to be of the kind investigated by Alper and Gillies, and we have to consider that chemical control and metabolic control are interrelated variables as regards radiation induced loss of reproductive integrity.

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## DISCUSSION

EIDUS: At what doses was there a bend in the dose curves in the experiments under anaerobic conditions?

GRAY: The lines were straight throughout.

EIDUS: I would like to point out that in well-known experiments by Dr. Hollaender on *E. coli* the dose curve under anaerobiosis after the initial bend runs, over several orders of magnitude, parallel to the first curve characteristic for the aerobic conditions, whereas in your experiments there is always a divergence of the curves. What is the explanation for this difference in the results?

GRAY: For the cell suspensions we have used, the form of the curves may differ slightly, but in general they are very similar.



TUMERMAN: I would like to point out the possibility that the important role of the water in radiobiological effects is due not only to the formation of the radiolysis products, but also to the fact that water has a structure, damage to which may contribute to the formation of the long lived triplet states.

PASSYNSKY: Probably with the aid of the micro-impulse technique Dr. Gray used it would be possible to determine the minimum time for the interaction of oxygen with the irradiated substance, which ought to depend on oxygen pressure.

ALEXANDER: I cannot agree with Dr. Gray that dose-response curves can be used to give quantitative information concerning the nature of the initial lesion in cells. There is now abundant evidence that every type of radiation lesion is capable of restoration after irradiation and that the magnitude of this restoration can be altered experimentally. Even if no special steps are taken to effect restoration, some is always taking place following irradiation. Moreover, it seems highly probable that radiation is also affecting the repair mechanism and this cannot therefore be assumed to introduce a constant factor which merely affects the size of the target calculated. The probability that ionization will produce a particular lesion is thus not governed only by "target size" considerations, but by a whole host of post-irradiation metabolic factors quite apart from protection and energy transfer phenomena which smear out the size of the target. In this complex chain of events, there would seem to be no direct or constant relationship between the end-effect and the initial act of ionization such as is necessary for the interpretation of dose-response curves along the lines you indicated.

The interpretation of the general shape of the curve in terms of multiplicity of events also seems to be unjustified since the effect of irradiation on the effectiveness of repair may well cause a curve to steepen progressively or to make an otherwise hyperbolic curve appear linear. I believe that it is quite likely that exponential dose-response curves of the type you showed for bacteria are the consequence of several factors acting in conjunction. This view derives support from the important work of Hollaender in which he showed that the shape of the dose-response curve of bacteria could be altered drastically by small changes in experimental conditions.

GRAY: I cannot understand why the fact that different dose-response relations are observed under different metabolic conditions should be a ground for rejecting an interpretation of each of these dose-response relations in terms of the number and probability of the initiating events which are radiobiologically significant under the respective metabolic conditions.

In my view the dependence of dose-response relations on metabolism is, in part, a natural consequence of the facts mentioned in the earlier part of Dr. Alexander's remarks, namely that many of the injuries sustained by cell components are repairable under certain metabolic conditions, but not under others. In part, it is probably a reflection of the fact that the significance, for the chosen criterion of biological damage, of an injury sustained by a particular organelle will depend on what metabolic activities the cell has to engage in after irradiation e.g. whether or not it has to adapt to a set of culture conditions different from those under which it was grown previously to irradiation. Properly used, the dose-response relation can be a valuable guide to the investigation of the mechanisms which underly the modification of biological response by post-irradiation metabolism.

By a dose-response relation I mean a curve, or its mathematical expression,

which gives a satisfactory fit to the experimental observations. The reliance which may be placed on this expression is determined by the accuracy of the fit and the range of dose over which the fit is maintained—as discussed fully by Zimmer ("Studien zur quantitativen strahlenbiologie", Steiner Verlag, Wiesbaden, 1960) and also by Dittrich ("Trefermischkurven" *Z. Naturf.* **15b**, 261, 1960). As explained by Lea, a given dose-response relation may, in principle, be interpreted either as an expression of the number of ionizing particles concerned in the initiation of the injury, or as representing a sensitivity distribution of the cell population to equal doses. When the form of the dose-response curve is simple, embodying a linear relation between the logarithm of the survivors and dose and a small extrapolation number (intercept between this line and the zero dose axis), I have no hesitation in choosing the former interpretation and inferring a low multiplicity of initiating events. To interpret a relation such as that which Dr. Dewey has observed for the proportion of survivors among an irradiated population of *Serratia marcescens* (shown as a lecture slide but unpublished), which is linear with dose over 9 powers of 10, in any other way seems to be unreasonable.

From the slope of the linear portion of the dose-response curve we obtain directly an estimate of the chance that at least one of the particles set in motion when a cell is exposed to unit dose of radiation will initiate the chain of events which lead to the biological effect under consideration. By the methods described in detail by Lea, and with the limitations pointed out by Lea, a knowledge of the slopes of the dose-response curves for different types of ionizing radiation can yield approximate information as to the size and shape of the critical organelle.

The purpose of a theory is to provide a basis for further experiments. If, having exercised due caution in deriving the best dose-response relation from the experimental data, we refuse to make the most obvious inference, we throw away a valuable means of deciding which, among the multitude of conceivable mechanisms, are the ones most worthy of further investigation.



# ACTION OF RADIATION ON PROTEINS AND NUCLEIC ACIDS IN SOLUTION AND AT INTERPHASES

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## SUMMARY

Measurement of the oxidation of the -SH groups of proteins, the linkages of  $^{35}\text{S}$ -methionine, the radiation destruction of DNA and many other methods allow us to establish the presence of molecular changes in thousands of irradiated molecules of proteins even for a dose of 400 to 500 r and in nucleoproteins for 20 to 50 r. However, the application of the statistical theory of the action of radiation shows that the primarily irradiated volumes in the cell only have the dimensions of a few molecules. When establishing the mechanism of biological "amplification" of the action of radiation it is necessary to take into account the particular significance of damage to the molecules of the biological macromolecules which enter into the composition of the intracellular surfaces of the section (cytoplasmic and nuclear membranes) and also the properties of the living cell as an open system for which the alteration of the transfer constant is of great significance. It has been shown that the appearance of chemical "cross-links" in monolayers of DNA substantially destroys the structure and increases the area of the monolayer, and radiation damage to the thin surface layers of RNA which separate the enzyme from the substrate (peroxidase-ascorbic acid,  $\text{H}_2\text{O}_2$ ) leads to considerable acceleration of their interaction. The variation caused by the damage of only several molecules in the surfaces of the section may be a source of all the subsequent biochemical disruptions and radiation damage to the living cells.

Living cells and organisms are constantly interchanging matter and energy with the surrounding medium, i.e. they form so-called "open systems". Therefore the theory of the action of radiation on living organisms must take into consideration the general properties of reactions in open systems. According to the theory of open systems (Burton, 1939; Denbigh *et al.*, 1948; Passynsky, 1957a) stationary concentrations of the components are determined not only by the rate-constants of chemical reactions, as in closed systems, but also, to the same extent, by the constants of the transfer of matter in the process of free diffusion or of penetration through membranes. Consideration of the constants of transfer, along with the coefficients of reaction rates is an essential feature of the theory of open systems which reveals the

indivisible relation between the spatial and temporal organization of metabolic processes.

The action of radiation on open systems, like that of other external factors, consists, in general form, in a disturbance of the stationary state of the system which, within a definite range of changes, can be compensated by the open system, while for more extensive changes it becomes impossible to establish a new stationary state and the system is degraded. The specific peculiarity of the biological action of radiations, however, when compared with other physical factors, is their ability to induce considerable physiological disturbances or even the death of the organism by small amounts of energy. The lethal dose for mammals (500 to 600 r) is known to correspond to the absorption of energy which, by heat equivalent, would cause a temperature rise of only some  $0.002^{\circ}\text{C}$ . But most of even this small amount of the absorbed energy does not have a lethal effect. For example, with a dose that produces an average of about 1,000 ionizations in each yeast cell, only some 10 per cent of the cells perish. It is beyond any doubt that so far as the effect of radiation is concerned the total amount of either chemically or structurally changed polymer molecules—such as proteins, nucleic acids—per cell is many times greater than that of changed molecules which effectively determine radiation damage to the cell.

By means of trivial physico-chemical methods (viscosity, electrophoresis, solubility, absorption spectra, etc.) the action of radiation on protein solutions can be detected only with very heavy irradiation doses, (75,000 to 100,000 r and higher). A new, isotopic method for the investigation of protein changes on radiation, has been developed in our laboratory. This is based on the ability of irradiated protein to show an increased binding of some organic substances, including  $^{35}\text{S}$ -labelled methionine (Passynsky *et al.* 1955). The high sensitivity of the isotopic procedure has enabled us to lower considerably the threshold of the observed action of radiations.

In most experiments 30 mg of protein was dissolved in 1 ml of borate buffer (M/15, pH 8.5), and 0.5 ml. of an aqueous solution of  $^{35}\text{S}$ -methionine (about 30,000 to 50,000 counts per min per ml of the mixture) was added to the solution. After irradiation the protein was precipitated with an equal volume of 10 per cent TCA, and the precipitate washed on a filter 50 times with a mixture of equal volumes of 10 per cent TCA and borate buffer; the constancy of the specific activity of the precipitate served to control the procedure. Thereafter the precipitate was dried and its activity measured on an end-window counter (evaluated per 10 mg of dry protein precipitate). It was shown in this way that appreciable molecular changes in protein (in 0.1 per

cent solution) could be revealed after as small an irradiation dose as 400 to 500 r.

The isotopic procedure was also applied to the detection of changes in serum albumin and serum globulin after  $\gamma$ -irradiation or after mixed neutron irradiation in a nuclear reactor, as well as to the X-irradiation of lipoproteins isolated from horse serum (Volkova and Passynsky, 1955). The data obtained are given in Table I.

TABLE I. *Thresholds of detectable radiation action*

Substances	Irradiation	Thresholds in thousands of r	
		by trivial phys.-chem. methods (viscosity, electrophoresis, etc.)	by isotopic method
Human serum albumin	X-rays	75 to 100	0.4 to 0.5
	$\gamma$ -rays	100	5
	Neutrons	250	5
Lipoproteins (from horse serum)	X-rays	1,000	50
Nucleoproteins (from the thymus)	—	0.05	—

Thus, the threshold of the action observed could be lowered 20-fold for lipoproteins and up to 200-fold for proteins.

When irradiating a high molecular weight nucleoprotein isolated from the thymus (N/P 3.3; 0.2 per cent solution in 1 M NaCl), no isotopic measurements were taken, since an appreciable radiation action was observed with as small a dose as 25 to 50 r by the decrease of the relative viscosity (Table I). At this dose the decrease of the relative viscosity was 4.5 per cent, while after 500 r it was 20 per cent (Volkova and Passynsky, 1955).

Thus, by means of sufficiently sensitive methods, the presence of definite molecular changes in the main classes of biological polymers can be revealed even with sublethal doses, with 400 to 500 r for proteins and 25 to 50 r for nucleoproteins. This result thus deprives the nucleic acids and nucleoproteins of the peculiar position ascribed to them by various theories of the biological action of radiations where these substances appeared to be the sole well-founded target for the action of sublethal doses of radiation, while other substances appeared either as inert media or as objects of secondary changes in the organism. On the contrary, protein molecules undergo change after these small doses, and it is evident that primary changes in cellular materials must be of a sufficiently general and wide-spread character. Hundreds and thousands

of ionizations arising in the cell at even comparatively low irradiation doses certainly do not pass without trace but remain in the cell in the form of hundreds of changed or damaged molecules of various substances, including such important ones as nucleic acids and nucleoproteins, proteins, enzymes, etc.

One of the main types of chemical change in protein molecules on irradiation seems to be the oxidation of SH-groups. In the work with Pavlovskaya (1956) we showed that the action of X-rays on 2 per cent aqueous solutions of crystalline egg albumin, gives an oxidation of SH-groups with an ionic yield of 1:1. The same result was obtained when dry preparations of crystalline egg albumin were irradiated; the total amount of oxidized SH-groups was  $6 \times 10^{18}$  per g protein, while the ionization number for the irradiation dose of  $5 \times 10^6$  r was about  $8.5 \times 10^{18}$  (Passynsky and Pavlovskaya, 1960). With a 20 per cent protein solution (assumed conditionally in the form of egg albumin) in the protoplasm the lethal dose of 600 r may lead to the oxidation of only 1 SH-group per 3,000 molecules. When taking into consideration the distribution of ionizations within the cell it may be assumed that the proportion of chemically changed protein molecules in the cell can hardly be greater than 0.01 per cent of the total number of protein molecules. Similar results are obtained, according to available data, for other chemical changes in protein molecules, such as the breakage of peptide bonds, deamination, etc. when doses of the order of 600 to 1,000 r are used. It seems that the transition of a great number of protein molecules to the excited or activated state at the expense of the absorbed radiation energy, which is accompanied by a re-grouping of some of the links in polypeptide chains and by corresponding structural changes, is of a considerable importance. These changes result in the increase of the aggregation of particles, decrease of solubility, activity augmentation of functional groups of protein, revealed, in particular, in the experiments described above by the increase of  $^{35}\text{S}$ -methionine binding.

It should be noted that the effects of the oxidation of SH-groups in protein molecules (in which these groups are the most radiosensitive areas) can hardly be explained by the formation of organic radicals. On irradiation of dry trypsin with fast electrons or  $\gamma$ -rays in oxygen, Alexander (1957) observed an increase of inactivation which he interpreted by the formation of  $-\text{RO}_2$  radicals from the primary  $-\text{R}$  ones, without, however, quantitatively analysing this suggestion. In our work (Passynsky and Pavlovskaya, 1960) the number of oxidized SH-groups and that of radicals measured by the ESR method was compared for one and the same preparation of dry egg albumin under the



same conditions of  $\gamma$ -irradiation *in vacuo*. It appeared that up to 45 SH-groups were oxidized per radical on irradiation *in vacuo*. On irradiation in air this discrepancy increases, since the number of peroxidized radicals (after the addition of  $O_2$ ), according to Alexander, is equal to that of primary radicals, while the number of oxidized SH-groups under these conditions increases twofold. This quantitative discrepancy is so considerable that the direct oxidation of SH-groups by radiation can hardly be explained by the formation of free organic radicals through the breakage of valency bonds, since in this case a closer correspondence between them and the number of oxidized SH-groups should be expected. The suggestion of Alexander (1957) on the role of molecular  $O_2^-$  ions in the oxidative effects observed seems to be more likely.

However, it may be expected that lethal or sublethal radiation doses (500 to 1,000 r) will lead to the appearance in the cell of hundreds or thousands of protein or nucleic acid molecules changed to a greater or lesser degree in chemical or structural respects.

Moreover, the primary inactivated volumes in the cell calculated according to statistical theory of the action of radiation have in many cases the size of only one or several molecules. These calculations are usually made on the grounds of the so-called "target-theory" which, in its physical basis, gives just the method of calculating the value of some inactivated volume with respect to various conditions of radiation (kinds of radiation, intensity of the dose, etc.) without defining the nature of this volume. Within this range the application of this theory does not have serious objections. In a paper by Pollard and his co-workers (1955) it was shown that statistical theory enabled one to determine directly by means of radiation the molecular weight of enzymes, hormones, viruses, etc., in a good agreement with the results of other experimental procedures. In our laboratory similar results have been obtained for preparations of lipoxidase (Budnitzkaya *et al.*, 1956) and insulin (Volkova *et al.*, 1957).

Thus the fundamental question of radiobiology is why does the damage to only a few molecules or of some cell portion of only molecular size lead to the death of the cell, while damage to hundreds of similar molecules in the cell produces no lethal effect, or, from the point of view of the theory of open systems, how can damage to a few molecules bring about essential disturbances in the stationary state of the cell?

The investigation of these questions was mainly directed in the literature to the establishment of specific structural changes arising after irradiation of the molecules of such biochemically important substances

as proteins, nucleic acids, lipids, corresponding proteins, etc. Despite the significance of this work, it should be noted that in essence they did not take into consideration the specificity of cell structures and the complicated heterogeneity of the internal structure of the protoplasm. Primary mechanisms of action of radiation were often analysed as if the cells were just a homogenous solution of various substances but in fact it is the complexity of the intracellular structure that underlies the qualitative peculiarities in the action of radiation on the living cell in comparison with its action on isolated cell substances and separates true radiobiology from radiation chemistry of complex molecules.

Current theories of radiobiological action associate the effect of the action of radiations with the damage of either nuclear structures (chromosomes), or microscopic cytoplasmic structures (mitochondria microsomes, etc). Chromosomes being unique cell structures, the first group of theories makes it possible to explain the death of cells with a "target" volume of molecular size, and the genetic effect of radiation, but it does not allow for physiological changes in cells after radiation and for many other radiobiological phenomena.

Considerable dependence of the number of chromosomal aberrations on the dose intensity, temperature and kind of irradiation, as well as many instances of their deviations from the exponential, show that they themselves may be of a secondary character (Bacq and Alexander, 1956). The second group of theories (Bacq and Alexander, 1956) attributes a major role to the damage of structurally-conjugated enzymic systems in mitochondria, microsomes, etc. These theories are also of certain interest; they encounter difficulty, however, in the fact of the multiplicity of mitochondria and microsomes in the cell. A sharp increase of radioresistance in polyploid cells, the chromosome number of which is only two to four times that of the normal, shows that in the presence of scores or hundreds of parallel functioning structures it is in fact very difficult to explain the high sensitivity of cells to the action of radiations by the damage of several molecules in one of these structures. One of the attempts to take into consideration the influence of intracellular structure in the light of the theory of open systems is the elucidation of the possibility of destroying the stationary state in the cells through essential change in diffusion parameters of the cell brought about, however, by damaging a small number of molecules.

It is evident that the cause of such an effect cannot consist in the destruction of observed structures formed by thick polymolecular membranes or films, since the destruction of several molecules in them would not essentially change their coefficient of permeability. It would seem that breaks in the structure of thin monomolecular or bimolecular



intracellular interphases in which a destruction of but one or two molecules can directly change the permeability of the layer, are more likely.

It is well known that the protoplasm is characterized by a considerable heterogeneity and multiphase nature of its inner structure. At the interphases of media of different composition thin molecular layers necessarily arise which are oriented at the interphase and contribute to the spatial organization of metabolic reactions.

The presence in the protoplasm of some inner interphases (ergastoplasmic membranes) forming in the protoplasm a number of internal zones or areas which sometimes sharply differ in pH or redox-potential values may be of great importance for the sequence of metabolic reactions. In this case damage to only some molecules in the bimolecular interphase switch on a chemical potential difference of much larger volumes or of relatively considerable numbers of components of bordering media, similar, for example to the situation in which a small hole in a dam can lead to the ultimate levelling of the very large water masses on both sides of it. The role of the change in the permeability of membranes has already been stressed by Bacq and Alexander (1956), but these authors meant mainly the membranes of mitochondria, while, in our opinion (Passynsky, 1957b), an essential change in the constants of the transfer in the cell as a whole which is required by the theory of open systems should rather be expected when one takes into consideration the molecular heterogeneity of the structure of the protoplasm. A disturbance of the normal sequence of substrate transfer in a number of enzymic conversions with a possible switching off of a number of reactions at once can be of a more specific or unique character than the damage of one or two enzyme molecules in one of the many hundreds of mitochondrial particles each of which contains hundreds of enzyme molecules.

The mathematical formulation of the target theory thereby preserves its significance both for the case when the primary inactivated volume actually corresponds to the size of molecules, and those cases when it corresponds to the size of a membrane (for example, in the work of Zirkle and Tobias (1956),  $r = 800 \text{ \AA}$ ,  $\alpha = 0.26\text{--}2.1 \text{ \AA}$ ). At the same time a direct relation between the inactivated volume and metabolic processes and the physiological state of the cell is evident.

From the same viewpoint some other fundamental problems of radiobiology can be interpreted (direct and indirect action, analogy with radiomimetic substances, activation of some enzyme systems on irradiation, influence of different kinds of radiation, etc. (Passynsky, 1957b).

X-ray structural investigation of thin films ( $300\text{--}400 \text{ \AA}$ ) of crystalline

polymers, e.g. polyethylene, irradiated with a beam of fast electrons (Karpov and Zverev, 1955) was carried out. The formation of but one chemical bond or "cross-link" between molecular chains as a result of contraction of the distance between them from  $4.5 \text{ \AA}$  to  $1.54 \text{ \AA}$  was shown to bring about a disturbance of the regularity of structure at a distance of scores of atomic groupings as a result of the transmission of tensions arising along molecular chains. Formation of double-bonds in an irradiated polymer acts in the same fashion. It was pointed out by these authors that a relatively small number of strong distortions of the lattice in polyethylene crystals as a result of radiochemical reactions in some 1 per cent of polymer links results in a conversion of the bulk of the substance from the crystalline to the amorphous state.

In our work with Tongur (1960) the action of transverse "cross-links" in a monomolecular layer of DNP (deoxyribonucleoprotein) on the structure of the monolayer was studied. The DNP monolayer was obtained on a Langmuir balance by applying microdrops of an 0.04 per cent DNP solution (mol. weight  $4.5 \times 10^6$  and  $8 \times 10^6$ ; N/P—3.8) in 1 M NaCl to a 38 per cent aqueous solution of  $(\text{NH}_4)_2\text{SO}_4$ . This lower phase was chosen in order to decrease the solubility of the nucleoprotein applied to facilitate the conditions of its spreading. The thickness of the DNP monolayer was about  $23 \text{ \AA}$ . Formation of transverse "cross-links" was achieved by the addition of 2 per cent formaldehyde to the lower phase. It was shown that without formaldehyde in the lower phase pressure-area curves which characterize the mechanical properties of the monolayer were rather constant for native and degraded (down to  $M = 20,000$ ) DNP preparations, while the value of the monolayer area was, respectively,  $0.36$  and  $0.30 \text{ m}^2/\text{mg}$  (some decrease of the area is due to the dissolution of a portion of the most altered molecules in the lower phase). On the contrary, in the presence of "cross-links", i.e. in the lower phase with formaldehyde, stable distortions of the monolayer structure arise, accompanied by an appreciable increase in the area/mg. For example, for native DNP the area of the monolayer increases from  $0.34$  to  $0.43 \text{ m}^2/\text{mg}$ , for the treated DNP from  $0.26$  to  $0.36 \text{ m}^2/\text{mg}$ ; the monolayer being compressed, it remains in a somewhat expanded state. This result, which is very similar to the data already mentioned for thin films of polymers, shows that radiation-induced formation of "cross-links" between polynucleotide chains in DNA and DNP monolayers leads to stable distortions of monolayer structure which, by their very nature, must result in a change in the permeability of these thin layers.

The influence of this factor on the course of enzymic reactions was studied by Passynsky and Volkova in the following model system. A preparation of crystalline peroxidase, in the form of a fine dry powder,

was suspended at 4 to 5°C in 0.1 per cent RNA solution (Merck preparation,  $M = 26,000$ ) in acetate buffer (0.1 M; pH 3.7). Under these conditions each grain of enzyme powder was covered with a thin surface layer of ribonucleoprotein (RNP) which enveloped the grain and inhibited its further dissolution. The suspension thus obtained was centrifuged and the supernatant poured off and replaced by the same buffer, the suspension was then stirred again and the process repeated two or three times to wash away all the excess RNA. The pure enzyme suspension in acetate buffer thus obtained was stabilized by a thin surface layer of RNA with particles of a diameter of 0.4 to 1.0  $\mu$  (Fig. 1). The nitrogen and phosphorus contents were determined. The N/P ratio in the precipitate of the suspension was 6.5; since all the phosphorus belonged to RNA, while the nitrogen contributed 13.2 per cent in protein and 14.5 per cent in RNA, then from the values of N and P the RNA

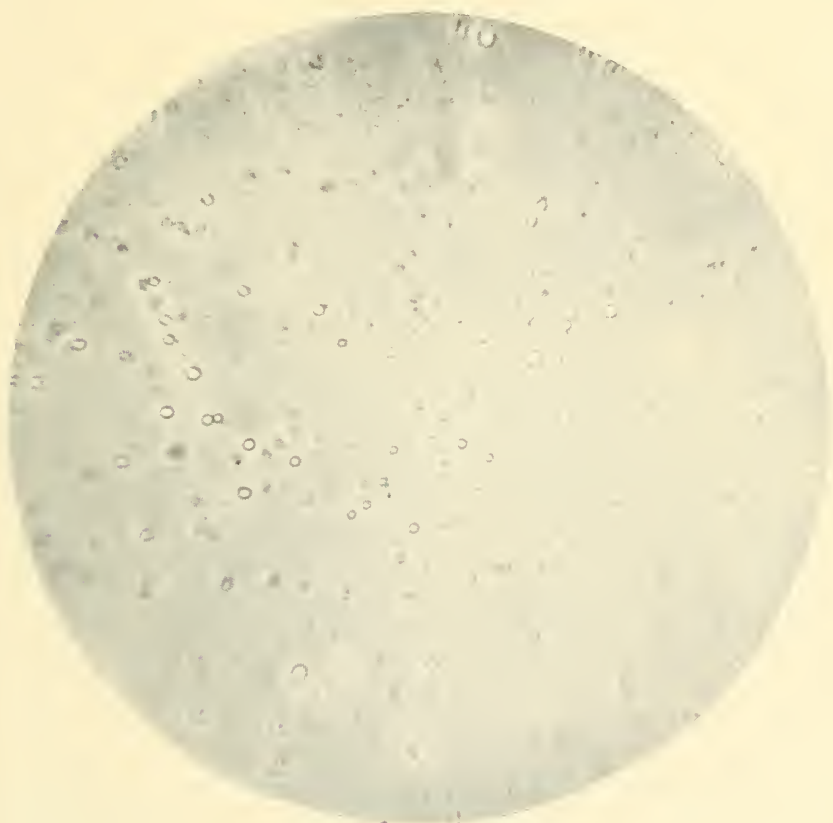


Fig. 1

content could be calculated and the thickness of its layer on the particles. For example, with a mean diameter of particles of  $0.4\mu$  the thickness of the surface RNA layer was  $160\text{ \AA}$  (Fig. 2) or about 8 RNA monolayers.

A known amount of ascorbic acid and  $\text{H}_2\text{O}_2$  in equimolar ratio was

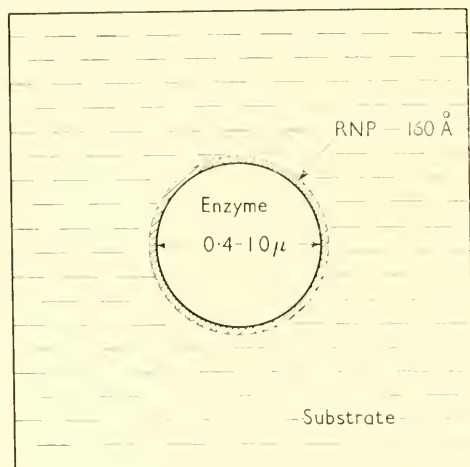


Fig. 2

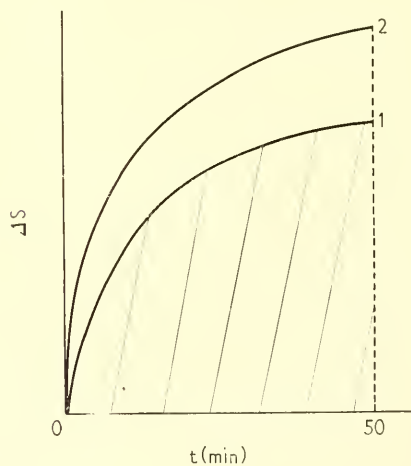


Fig. 3

added to half of the enzyme suspension stabilized by the surface RNA layer and the kinetics of enzymic oxidation of ascorbic acid were determined on the contact between the enzyme and the substrate through

the thin separating RNA layer (Fig. 3, curve 1). The second half of the enzyme suspension in acetate buffer was exposed to X-irradiation with various doses; thereafter the substrate was added to this half of the suspension and the rate of enzymic oxidation of ascorbic acid through the RNA layer damaged by radiation was determined in the same way (Fig. 3, curve 2). In the second case the oxidation of ascorbic acid always proceeded faster. The activity of pure aqueous solutions of peroxidase changed but little with the doses of irradiation used, (28,000 to 70,000 r); besides, inactivation of the enzyme could only lead to the slowing down of the rate of ascorbic acid oxidation but not to an increase in this rate. In control protein RNA suspensions, where the enzyme preparation was replaced by human serum albumin or on mixing RNA and ascorbic acid solutions, radiation did not alter the course of spontaneous ascorbic acid oxidation (Fig. 4), very much unlike

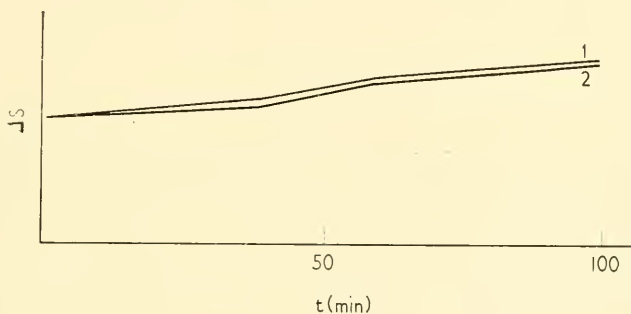


Fig. 4

the course of the curves in Fig. 3. Therefore, it seems that the acceleration of the enzymic oxidation of ascorbic acid in irradiated peroxidase-RNA suspension could be regarded as a result of an increased permeability of the damaged thin RNA layer separating the enzyme and the substrate, and of an acceleration of the diffusion stage of enzymic reaction. Applying as the measure of acceleration of enzymic reaction the ratio of the areas of curves 2 and 1 in Fig. 3 (and in similar experiments with other doses) one can plot a further curve (Fig. 5) characterizing the dependence of the effect observed on the dose of radiation. The dotted part of the curve in Fig. 5, corresponds to scattering of points (about 7 per cent) in the control, in various unirradiated enzyme suspensions. Thus, the dose of about 18,000 r can be seen to be the threshold of the experimentally observable effect. It may seem that this threshold lies above the doses of biological importance; it should be taken into consideration, however, that in the suspensions tested the



surface layer consisted of 8 molecular layers of RNA, i.e. of those where scores of damages can arise in various places attenuating the total effect of the permeability increase which is undoubtedly of a statistical character. In a mono- or bimolecular layer each damage must considerably change the permeability of the layer; therefore it can be expected

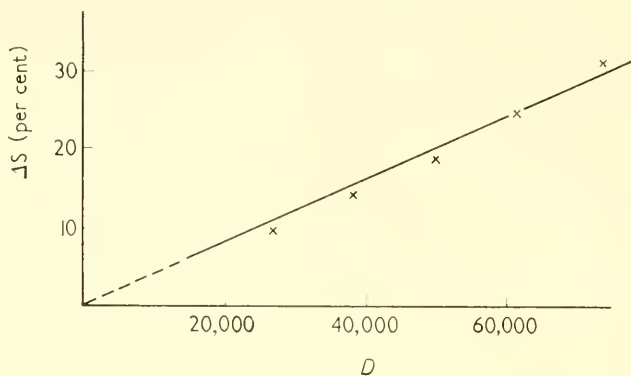


Fig. 5

that the effect discussed can take place in mono- and bimolecular layers directly within the limits of biologically important doses. When obtaining enzyme-substrate systems with finer molecular separating layers of different nature, a considerable lowering of the threshold of the action observed can be expected.

At any rate, from the viewpoint presented, which was developed in detail earlier (Passynsky, 1957b), it is evident that the key, specific importance of certain damage to molecules in the cell can be explained not by some unique peculiarities of their structure but by their participation in molecular interphases. In the light of the theory of open systems the damage of a few molecules in cell interphases can lead to a considerable change in the constants of transfer and in the destruction of the organization of biochemical processes in the cell even in those cases when the bulk of enzyme and substrate molecules remain intact (for example, in the action of sublethal doses of radiation). It should be noted that similar conclusions were drawn in our laboratory from the study of enzymic oxidative processes in living objects, in leaves of various plants (Budnitskaya *et al.*, 1956; Passynsky and Vyrovetz, 1959). The source of biological strengthening of the action of radiation can thus reside in the specificity of the fine intracellular structure.

It seems that this factor may play an important part in the mechanism of biological strengthening of the action of radiation and, therefore, in the theory of the biological action of radiation.



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## DISCUSSION

BACQ: What is the relative efficiency of the  $\alpha$ -particles and X-rays with regard to the enzyme system you have studied?

PASSYNSKY: Up to now X-rays alone have been studied. Comparison with the effects of  $\alpha$ -particles is undoubtedly of the greatest interest, since until now different models always differed from the living cell by the relative efficiency of different irradiations.

GRAY: How do the radiation effects depend on the thickness of the RNP layer around the enzyme particle?

PASSYNSKY: We tried to obtain layers as thin as possible, but the influence of the irradiation up to now has only been studied for the minimum thickness obtained—about 8 molecular diameters.

ARDASHNIKOV: What is the explanation for the irradiation dose effect?

PASSYNSKY: At a given dose in every monolayer an equal number of the structural lesions would be produced, irrespective of the number of the successive layers. But the effect of these lesions on the permeability of a separating layer of course would not be the same, if the layer's thickness varied. In a layer consisting of one row of molecules every lesion would change the permeability; whereas in the case of a multi-molecular layer it is necessary that lesions of different layers should form a kind of channel, and that is an unlikely occurrence. Just because of this it may be expected that the sensitivity of the system to irradiation would increase rapidly.



# ELECTRON SPIN RESONANCE (ESR) INVESTIGATIONS ON RADIATION-INDUCED CHEMICAL EFFECTS IN BIOLOGICAL SPECIES

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## SUMMARY

The ESR method may be used to investigate radiation-produced unpaired electrons in lyophilized biological structures. The authors have studied nearly all the amino acids, a number of di- and tripeptides and various proteins, nucleoproteins and tissues.

The number of free radicals produced in  $\gamma$ -irradiated lyophilized proteins is some two or three orders of magnitude less than in amino acids and peptides. It is suggested that "conductive channels" may exist and that electrons can migrate along these, thus "healing" the injuries.

The appearance of a new method of investigation, especially if based on exact theory, often leads to considerable progress. This is exactly so in the case of the ESR method, which was invented by the Soviet physicist, Zavoysky, in 1944.

There are a number of articles on the theory of ESR and its experimental techniques (Frenkel, 1954; Gordy, *et al.*, 1955; Ingram, 1955, 1958; Van Vleck, 1948; Semenov and Bubnov, 1959). These problems will not, therefore, be discussed.

It is known that the ESR method permits us to obtain the following information about the substances investigated;

1. The presence of unpaired electrons at concentrations of about  $10^{-11}$  to  $5 \times 10^{-12}$ M per gram, or, in other words,  $10^{12}$  to  $10^{14}$  unpaired electrons in the sample;
2. A sufficiently correct quantitative estimation of the concentration of unpaired electrons (by comparison with standards);
3. The analysis of the form and the width of ESR spectral lines permits important conclusions to be drawn about the interaction between unpaired electrons and the surrounding atoms; the influence of structural particularities of the substance being investigated on the

behaviour of unpaired electrons; the magnitude of exchange interactions and the degree of delocalization of such electrons, and other important parameters;

4. The analysis of hyperfine ESR structure (resulting from the interaction of unpaired electrons and nuclear magnetic moments of surrounding atoms) allows us to make the structure of paramagnetic particles clear.

Free radicals formed by ionizing radiations have unpaired electrons. For this reason the ESR method has now become one of the most widespread and effective methods of investigating initial radiation-induced chemical effects. The investigation of the kinetics of free radical formation, while the dose rate, the energy of radiation and the intensity of emitting sources are varied; the study of the temperature effect and the effect of oxygen; the study of the effects of humidity; the investigation of anti-radiation compounds; the determination of the quantum yields of radiation-induced chemical reactions; the evaluation of the recombination energy of radicals by use of kinetic curves; all these problems and others can be solved by means of ESR spectroscopy.

Hutchison (1949) was the first to observe the ESR spectra of irradiated organic compounds. He irradiated alkyl halides with neutrons and found them to form  $\tau$ -centres, which give ESR signals.

ESR investigations of irradiated compounds, important biologically, were begun in three countries independently: by Combrisson and Übersfeld (1954) in France, by Gordy and his collaborators (1955, 1958) in the U.S.A. and by us in the U.S.S.R. (Blumenfeld, 1958; Blumenfeld and Kalmanson, 1957a,b,c; 1958a,b; Kalmanson and Blumenfeld, 1958).

Combrisson and Übersfeld irradiated amino acids in an atomic pile directly. The wide energy spectrum of the source, the high total dose rate and the high intensity of the irradiation lead to extensive destruction of the amino acids. In such conditions the ESR spectra of the free radicals were rather similar with only a slight hyperfine structure.

Since 1955 more complete and interesting investigations of the ESR spectra of irradiated biological materials have been carried out in the U.S.A. by the group headed by Gordy, who investigated such important irradiated biological compounds as amino acids, proteins, hormones, vitamins, fats and nucleic acids.

The most interesting and unexpected result of this work proved to be that although the amino acids, of which proteins are constituted gave ESR spectra after irradiation that were characteristic for each amino acid, irradiated proteins always gave rise to only two types of signal: either to a doublet with a splitting of 15 to 16 oersted, or to a

signal characteristic of compounds containing sulphur. The doublet was observed in proteins that did not contain sulphur, such as, for example, collagen.

Signals characteristic of irradiated sulphur-containing amino acids, were observed with those irradiated proteins which contain a considerable amount of sulphur, such as wool, horn, hoof, and nails.

No other hyperfine structure in sulphur-containing proteins was found by Gordy and his collaborators although all these proteins contain enough protons and nitrogen nuclei in various positions. It is known that the magnetic moments of such protons and nitrogen nuclei are responsible for the appearance of the hyperfine structure of irradiated amino acids.

Gordy was quite right in suggesting a possible migration of "injuries" along the protein molecules towards certain more vulnerable points whose properties in this respect, however, were not made clear. Gordy gives no explanation of the "migration of injuries" in his early work.

In his later work Gordy develops various assumptions of the possible radio-protective role of sulphur-containing compounds. Such studies are justified and to some extent are connected with the numerous radiobiological investigations on the anti-radiation effect of sulphur-containing substances.

In spite of numerous experimental data and a quite profound theoretical evaluation of these data from the point of view of quantum chemistry and the ESR theory, we think that Gordy's work has some defects. First, no quantitative estimation is given of radical yields for irradiated biological structures of different complexity and in different native states. The second defect, apparently due to the physical rather than biological treatment of the problems being studied, is that Gordy's group often use proteins without clearly defined biological properties.

So far as radiobiology is concerned some research papers published by the Swedish scientists Lars and Andreas Ehrenberg (1958) together with the noted German radiobiologist Karl Zimmer (Zimmer, 1960; Ehrenberg and Zimmer, 1956) are far more interesting. In these papers ESR spectra of irradiated seeds and sprouts of different water content were investigated.

The authors gave a quantitative estimation of the radical yield as a function of the radiation dose, and also traced the rate of decay of free radicals after the irradiation as a function of the percentage of water of the samples.

The main results of the Ehrenbergs' and Zimmer's work are:

1. A linear dependence of the free radical yield on the radiation dose was established.



2. A protective action of water was discovered; the yield of radicals was halved as the water content of the irradiated seeds increased from 3 per cent to 10 per cent. This protective action, according to the authors, is connected with the greater possibility of recombination between free radicals as the water content in the samples is increased.

3. Some protective action of water was also observed by the authors in experiments on the germination of irradiated seeds of different humidity. In these experiments seeds of lower water content perished if irradiated by smaller doses.

4. ESR studies of the rate of decay of free radicals in samples of different water content showed an exponential decrease, the coefficient before the exponential factor being larger for samples of higher water content.

The work of the Ehrenbergs and Zimmer successfully combines the possibilities of new physical methods and ordinary radiobiological experiments, and thus help to approach one of the most debatable problems of radiobiology—the problem of the direct and indirect (with water present) action of irradiation.

Any theory that takes into account the participation of the low molecular products of water radiolysis must consider the extremely high reactivity and short life-time of these products at the temperature of living cells. It would be right to mention, that according to the ESR data atomic hydrogen can be observed in irradiated ice only at liquid helium temperatures, and the free OH radical only at liquid nitrogen temperatures.

We shall now proceed to give an account of our own experiments and of some general conclusions which we believe can be drawn from them. First of all it must be pointed out that we used ionizing radiation just to obtain and accumulate unpaired electrons in biological structures of increasing complexity and different native states and not at all for the investigation of the initial radiochemical aspects of radiobiological problems. We hoped that the investigation of ESR spectra in such a system would help us to find out what structural peculiarities give rise to the surprising activity of biological structures.

We proceeded from the general hypothesis that the important role of free electrons in a number of most important biological processes is connected with energy migration within the biological structures. Initially we supposed that enzyme action and muscle contraction belonged to such phenomena. It is quite clear, however, that all our results are also closely related to the nature of initial radiation-induced effects.

Having these general considerations in mind in 1955–1957 we systematically investigated a wide range of irradiated biological objects:



nearly all amino acids, a number of di- and tripeptides and various proteins and tissues, some of them biologically active, and some of them destroyed beforehand by boiling.

The source of irradiation was cobalt-60. The samples were irradiated with a dose of  $10^6$  to  $10^7$  roentgens. The amino acids were investigated in the crystal state, while proteins and tissues were in the form of lyophilized specimens retaining a maximum of their native properties. Quantitative measurements were carried out by comparing the areas of a standard signal with that of the signal from the sample being tested.

The structure of the free radicals formed by irradiating amino acids especially were not investigated in detail. However, in a number of cases we were able to reveal their structure sufficiently correctly and completely. A more detailed study of the structure of free radicals, formed by irradiating complex amino acids would require a special investigation with additional modification of the amino acids, for instance the introduction of isotopic atoms such as deuterium, which has a different nuclear spin from that of a proton and which can be introduced into certain groups in amino acids. We feel, however, that such additional complications would probably not help us much to solve the main problem of ascertaining the peculiarities of the macrostructure of the most important biopolymers, proteins and nucleic acids.

The main experimental results may be considered:

(i) Dry specimens of amino acids as a rule produce intensive ESR spectra with a width of tens or hundreds of oersteds and with a clearly defined hyperfine structure, due to the interaction of unpaired electrons with protons and nitrogen nuclei, which are part of the free radicals produced. With a dose of about  $10^7$  r the yield of free radicals is about  $10^{19}$  paramagnetic particles per gram. It is possible to assert that every ionization event in the amino acid leads to the production of about one free radical. As a rule, most amino acids evolve ammonia and  $\text{CO}_2$  following irradiation, and sulphur-containing amino acids evolve hydrogen sulphide. The spectra of some simple irradiated amino acids can be interpreted as spectra of amino acid fragments which remain after such decomposition products have been removed. However, the unpaired electron of a sulphur-containing compound can be considered to be localized near the sulphur atom; this results from the value of the  $g$ -factor for such compounds.

It is worthwhile to note that in irradiated dry crystalline preparations of amino acids ESR spectra, and that means free radicals also, have been kept unchanged at room temperature (and in the presence of air) for more than four years already; however, if the crystals are dissolved in water the radicals vanish immediately.

(ii) In the ESR spectra of irradiated native proteins and lyophilized tissues (60 to 80 per cent of proteins) two special features may be discerned. First, the number of free radicals produced in proteins and tissues is from one to three orders of magnitude less than in amino acids and peptides (with equal doses of  $\gamma$ -irradiation). Second, the signal is not a superposition of ESR spectral patterns (this might be expected since the energy of  $\gamma$ -quanta is quite sufficient to break any peptide bonds in proteins), but is usually a single narrow peak with a half-width of 4 to 10 oersted and without any hyperfine structure.

The position and the width of the signal is usually the same as that for ESR spectra of enzyme specimens frozen and lyophilized at the moment the enzyme action takes place. The narrowness of the signals cannot be explained by exchange interactions, because of the small concentration of unpaired electrons. We must admit a translational mechanism for the narrowing (Anderson 1954).

The small intensity of signals in irradiated proteins and the absence of hyperfine structure, in comparison with signals from irradiated amino acids, are of special interest to radiobiologists.

In the case of irradiated amino acids and peptides the ESR signal is due to "holes" with unpaired electrons, or to neutral free radicals arising when bonds are broken homogeneously.

However, from the point of view of our hypothesis about the existence of energy levels of a molecule as a whole or, as we called them, "conductive channels", it may be suggested that in native proteins eliminated electrons can re-enter the previously mentioned "bands", formed by an orderly net of hydrogen bonds, and return along them to the "holes", recombining with them and "healing" the injuries.

This hypothesis about the role of regular hydrogen bonds in creating "conductive bands" and in increasing the radio-resistance of proteins, was investigated in special experiments.

It is known that heat denaturation sharply disturbs an orderly system of hydrogen bonds, and makes it chaotic. When denaturation is sufficiently complete proteins lose their biological activity completely. A number of protein preparations were exposed to heat denaturation before lyophilization and irradiation. In all cases intense signals were obtained with clearly expressed hyperfine structure, characteristic of free radicals with localized electrons, instead of a weak narrow singlet. By increasing the amount of denaturation, an increase of radical yield by one to three orders was produced.

We suppose that the disturbance of the secondary structure of protein molecules during the denaturation leads to the disappearance of "conductive channels" along which the electrons ejected by the

radiation might recombine with "holes" and this is shown by the increasing concentration of unpaired electrons in the sample, such unpaired electrons being already localized.

In 1958-1959 the ESR spectra of irradiated nucleic acid compounds was investigated. This work was carried out in collaboration with Passynsky and Shen-Pei-Gen (Shen-Pei-Gen *et al.*, 1959).

Nucleic acids and their components were irradiated under the same conditions as before. We also irradiated nucleoproteins and some artificial complexes of nucleic acids with proteins and other compounds.

As in the investigations of amino acids and proteins we found a distinct dependence of free radical yield on the complexity of the structures. For instance irradiation of nucleic acid bases and ribosides with a dose of  $10^7$  r gave a free radical yield of  $10^{18}$  to  $10^{19}$  particles per gram. The free radical yield is one to three orders less for high molecular weight preparation of nucleic acids.

The most important result, from the point of view of radiobiology, is that although the radical yield per gram for low molecular weight compounds is much higher than for those of high molecular weight, the number of damaged molecules per ionization event is, however, much higher (50 to 70 times) because of the great dimensions of the nucleic acids.

This conclusion is in excellent agreement with other purely biological experiments in radiation cytology and genetics, which showed that a disturbance in even one section of a high polymeric nucleic acid, which is a part of the chromosomes, can lead to non-reversible and even lethal damage to the cells. We can explain the small radical yield in irradiated high-polymer nucleic acids on the basis of our hypothesis about the existence of molecular "conduction channels".

It is interesting that in one of their last papers on the irradiation of nucleic acid compounds, Shields and Gordy (1959) also conclude that nucleic acids have semi-conductive properties.

In conclusion it is necessary to point out that all the experimental data given above were obtained on lyophilized, solid specimens. Because of this, direct comparison of these data with those for biological species containing much water is not quite correct. In aqueous media additional difficulties, due to the secondary action of short-lived active free radicals created by water radiolysis, can occur. However, we are sure that these special features of the ionizing action of radiation on biological polymers are due not to aggregation, but to properties of their molecular structure.

From this point of view molecules of protein and nucleic acid may be considered in aqueous medium as particles of a solid body. So we think

that, without taking into consideration the influence of short-lived radicals, the mechanism of the initial action of ionizing radiations on biological structures does not depend on their state of aggregation.

Some papers have recently been published, testifying to the specific role of molecular and supra-molecular order in the peculiar properties of biological structures, for example the work of Arnold and Sherwood (1957) on the semi-conductive properties of biological structures, as shown in particular by photosynthetic properties; our work on abnormal magnetic properties of nucleic acids and nucleoproteins (Blumenfeld *et al.*, 1959; Blumenfeld, 1959); and the work of Polonsky *et al.* (1960) and Duchesne and Monfils (1955) on the magneto-electrical properties of nucleic acids.

We believe that all these phenomena are due to the high degree of order of these materials. Evidently this high degree of order in biological structures conditions the peculiarity of their radiolysis, and is responsible for the characteristics of their ESR spectra.

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## DISCUSSION

BACQ: You have worked with absolutely dry proteins. Can you give us the exact temperature and oxygen pressure during the irradiation.

BLUMENFELD: Irradiation was carried out at room temperature. Oxygen pressure during irradiation was about  $10^{-3}$  mm Hg. Our samples were freeze-dried at a temperature of  $-50^{\circ}\text{C}$ . ESR spectra were studied both at room temperature and at lower temperatures, down to liquid nitrogen temperature. I did not dwell here on ESR spectrum changes in irradiated amino acids associated with temperature decrease: it could be the subject of a special report. As for an oxygen effect, it was practically non-existent as far as amino acids and low molecular weight compounds were concerned, but oxygen affected considerably the high molecular weight compounds, particularly proteins: the intensity of the ESR signals decreased considerably on admission of air.

As to the form of the ESR signals, since we did not perform experiments with irradiation at low temperatures but applied irradiation at a sufficiently high room temperature, we did not observe any changes in the form of the ESR signals.

ALEXANDER: In our experiments we have observed that when DNA from sperm heads is irradiated at a low temperature, a high yield of radicals occurs. After irradiation at room temperature there is a drop in the radical yield. Our explanation was that at high temperatures a recombination of radicals is possible due to molecular movements. Could your theory account for this phenomenon?

BLUMENFELD: Your observation that during irradiation the radical yield increases with the lowering of temperature, is a very interesting one. I believe that it fits in well with our conceptions. For uncoupled electrons to migrate through formed structures, it is necessary that some quite definite structural conditions be fulfilled, which, generally speaking, could be unfulfilled (not realized) in the structure of the proteins and nucleic acids. In order for uncoupled electrons (which are  $2p$  electrons), to migrate through the hydrogen bond system, it is necessary that on all the centres through which they migrate their wave functions be parallel.

This condition may be unfulfilled in molecules of the nucleic acids and proteins at equilibrium configuration.

ALEXANDER: May the signal be more intense at low temperatures?

BLUMENFELD: In order that semiconductivity properties become manifest, an orderly structure of the hydrogen bonds in protein and nucleic acid molecules is necessary. It is necessary that in proteins all the peptide groups by which migration occurs, should lie in the same plane. If they are oriented at right angles migration is impossible. If they are oriented at different angles, the probability of the migration is proportional to the cosine of the angle. At liquid nitrogen temperatures these conditions may be unfulfilled under equilibrium conditions for all the structure. At room temperature owing to vibrational and rotatory movements there always would be moments when different peptide groups are in the same plane and migration may occur.

PASSYNSKY: What is the significance of the  $g$ -factor in all the systems studied? Is it possible to relate the observed intensity of the signals only to the number of the uncoupled electrons, without taking into account their interaction?

BLUMENFELD: In all the cases for all the ESR signals in proteins and nucleic acids elicited by the ionizing radiation—I emphasize it—the  $g$ -factor of the



signal practically coincides with  $g$ -factors of the free electron except for several proteins with high sulphur content, which give signals with  $g$ -factor of 2.024, testifying to the localization of the uncoupled electrons on the sulphur atom.

I believe that in all the cases mentioned here the magnitude of the signal is determined only by the number of the uncoupled electrons, as in all the compounds with paramagnetic properties.

PASSYNSKY: To what extent are all-molecular electron levels utilized in proteins and nucleic acids unexposed to irradiations under their basic natural conditions?

BLUMENFELD: I believe that in all the proteins and nucleic acids there are potentially these all-molecular levels, but under the basic conditions of these compounds they are unpopulated. In the case of proteins they become populated when enzymatic processes occur, owing to the formation of the complex substrate-enzyme. Or they become populated as an effect of irradiation. But protein molecules as such do not possess populated all-molecular levels and from this standpoint are not semiconductors.

# THE ACTION OF X-RAYS ON INTRACELLULAR BACTERIOPHAGE FORMATION

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## SUMMARY

1. Inactivation of the capacity of *E. coli* B for phage T 3 by the action of soft X-rays was measured.

2. The dose-effect curve is biphasic. After small doses, rapid inactivation occurred, but the inactivation was markedly slower after large doses.

3. The capacity of irradiated *E. coli* B was affected by addition of chloramphenicol. On adding chloramphenicol to irradiated bacteria together with the phage during the logarithmic phase, the decrease in capacity was relatively smaller than might have been expected if the deleterious effects of the two factors were cumulative. In stationary cultures the protective effect of chloramphenicol was less marked.

The ability of bacterial cells to form phage was defined by Benzer and Jacob (1953) as the capacity. Earlier observations by Anderson (1944, 1948) showed that cells of *Escherichia coli* sterilized by large doses of ultraviolet radiation could still form phage T4. Later it was found that the capacity of *E. coli* for other phages was much more sensitive to the action of ultraviolet radiation (Benzer, 1952; Garen and Zinder, 1955; Labaw *et al.*, 1953; Tessman, 1956). Similar results were obtained with ionizing radiation (Rouyer and Latarjet, 1946; Tobin, 1953; Garen and Zinder, 1955; Pollard *et al.*, 1958). Recent measurements (Stent, 1958) have shown that, for phage T 2, a dose of 650,000 r is required to inhibit two-thirds of the bacterial phage-forming capacity.

A deeper insight into this remarkable phenomenon will not only elucidate, to a certain degree, the mechanism of the basic action of radiation on living matter but will also reveal the processes of phage formation.

In our laboratory the inactivation by X-rays of the capacity of *E. coli* for phage T3 has been studied. The immediate effect of ionizing radiation was compared with the delayed effect. In another series of experiments the protein synthesis of the irradiated bacterial cells was

stopped by chloramphenicol either before or after infection of the cells with phage (Herčík, 1959, 1960).

The bacteria were irradiated on broth agar, from which they were washed off either immediately after irradiation or 24 hr later. They were then tested for the ability to form phage by adding phage T 3 and determining the titre of the phages developed. The survival of the capacity after irradiation was determined from the ratio of phage yield of irradiated and of non-irradiated controls. To avoid formation of colonies out of surviving cells, the bacteria destined for the observation of the delayed effect were kept for 24 hr at a temperature of 6°C. This procedure was in accordance with that of Stapleton *et al.* (1953), who found that if cells of *E. coli* were cultured at temperatures between 4°C and 37°C after irradiation, the inhibition of division was the same at 4°C as at 37°C (with a maximum of recovery at 18°C.) By using this method with doses up to 20,000 r it was possible to avoid errors that would otherwise have occurred as a result of capacity in bacteria that had developed by proliferation of surviving cells.

The bacteria were irradiated with a tube of 60kV, 4 mA and a dose rate of 4,000 r/min. The range of doses used was from 4,000 r to 960,000 r. The survival of *E. coli* B corresponded to the customary value of 3,500 r for the half-value dose (one-hit curve). After a dose of 120,000 r only one cell in  $10^7$  is capable of forming colonies.

The results of this series of experiments are shown in Fig. 1. The inactivation of the capacity by ionizing radiation differs according to whether it is determined immediately after irradiation or 24 hr later. Immediately after irradiation the decrease in capacity in relation to the dose, on a semilogarithmic scale, is only approximately linear. This is in agreement with the results obtained by Pollard *et al.* (1958) for phage T 1. This is even more manifest after 24 hr. After small doses (4,000 r and 8,000 r) a sharp linear decrease in capacity occurs. The response to higher doses is quite dissimilar. There is also a linear decrease, but the drop in the capacity is much slower.

The explanation of this phenomenon is difficult. It should be taken into account that the inactivation of the bacterial capacity to produce phage is the result of the irradiation reaction which has its own course. It appears that recovery processes develop in the case of the immediate effect of high radiation doses (as a result of the long exposure period) which reduces the degree of inactivation of the capacity. On the other hand with the delayed effect the change in the capacity follows the course of the completed irradiation reaction and, therefore, relatively small doses of irradiation produce a large drop in the capacity. With doses higher than 80,000 r the bacterial capacity becomes more radio-

resistant. This probably indicates a second mechanism of phage formation. At the present time there is not enough evidence to support the view that there are two distinct mechanisms of phage formation

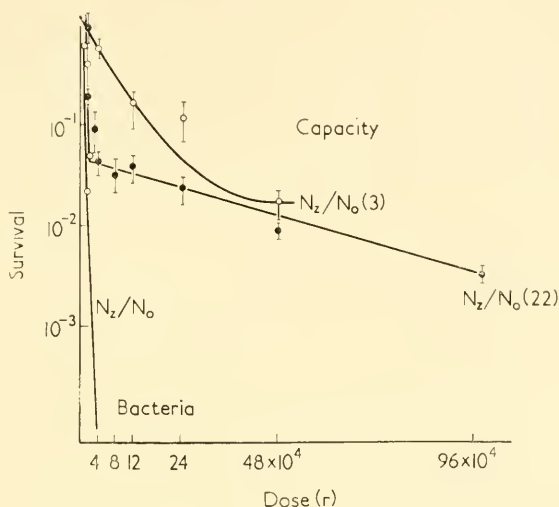


Fig. 1.—Effect of X-rays on the capacity of *E. coli* B to form phage T 3.

- Survival of the bacteria
  - Change in capacity immediately after irradiation.
  - Change in capacity 24 hr after irradiation.
- Numbers in brackets: age of the culture in hours.

with differing radio-resistance. However, several examples do exist where dose-effect curves are non-uniform, indicating processes with different radio-resistances.

Another possible explanation based on the non-homogeneity of the particular population of *E. coli* B resulting in two different types of capacity appears to be even less probable. The reason is that in such a case the inactivation curve of the colony-forming ability should be also biphasic, and this is not the case.

In looking for an explanation of the biphasic nature of the bacterial capacity, we tried to influence the phage production of the irradiated bacterial cells by chloramphenicol. It is known that chloramphenicol inhibits protein synthesis in *E. coli* B (Wisseman *et al.*, 1954). In *E. coli* B cells irradiated with X-rays or u.v., Gillies and Alper (1959) found a higher survival rate in cells incubated on agar containing chloramphenicol. Phage formation by bacterial cells is also influenced by chloramphenicol (Bozeman *et al.*, 1954; Crawford, 1957, 1959). It was,

therefore, to be expected that chloramphenicol would influence the production of phage in irradiated bacterial cells.

The action of chloramphenicol on the capacity of irradiated *E. coli* was studied in two series of experiments. In the first series, the chloramphenicol was added to the irradiated bacteria at the time of infection with the phage, or 15 min later at the end of the latent period. Bacteria were infected for the determination of the capacity immediately after irradiation or 22 hr later. In the second series of experiments the irradiated bacteria were exposed to the action of chloramphenicol for different times before determining the capacity.

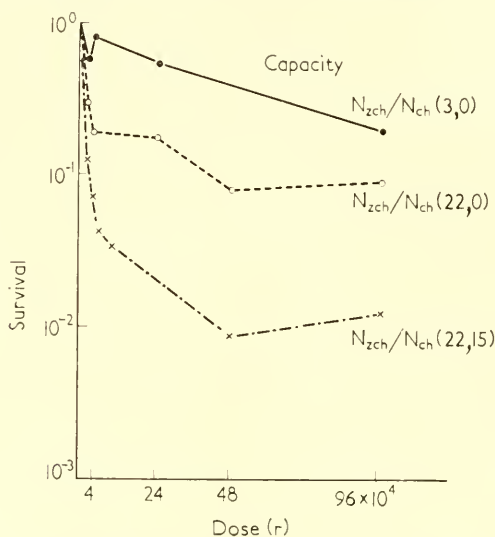


Fig. 2.—Relationship between capacity of *E. coli* B for phage T 3 in the presence of chloramphenicol after irradiation with X-rays.

$N_{ch}$ —capacity of non-irradiated bacteria treated with chloramphenicol.

$N_{zch}$ —capacity of irradiated bacteria in the presence of chloramphenicol.

Numbers in brackets: the age of culture in hours and the time in minutes at which chloramphenicol was added.

The toxic effect of chloramphenicol on the capacity of *E. coli* B was investigated without previous irradiation. It was stated that the addition of chloramphenicol during the logarithmic phase caused a marked reduction of capacity (222-fold), while during the stationary phase the reduction in capacity was about tenfold.

Under these circumstances the combined effect of chloramphenicol and radiation is bound to be manifested in a further decrease of the capacity. In general it may be stated that in the presence of chloramphenicol the capacity of the bacteria decreased with the radiation dose,



and that the biphasic character of the dose-effect curve was maintained. Very interesting results are found when the data are plotted in such a way that the capacity of irradiated and chloramphenicol-treated cells is compared with non-irradiated cells treated with chloramphenicol. The effect of irradiation on the capacity was lowest in the 3-hr culture to which chloramphenicol was added, together with the phage, immediately after irradiation. It has already been stated that the chloramphenicol itself has a high toxic effect; it would, therefore, be expected that heavy irradiation doses would lead to complete inactivation of the capacity. This is evidently not the case (see Fig. 2), and chloramphenicol must, therefore, have a restorative effect.

These experiments have clearly shown that chloramphenicol and radiation do not supplement one another in inhibiting the capacity of *E. coli* B to sustain phage growth but that under certain conditions they can be antagonistic to each other.

This problem was investigated in a second series of experiments with the aim of diminishing the toxic effect of chloramphenicol. The method of Gillies and Alper (1959), using cellophane carriers on which the bacterial culture was spread, was used. This method enabled us to remove the carriers, after certain time intervals, from the surface of the agar medium containing chloramphenicol. In these experiments, which have not yet been completed, it can be shown that a short stay of heavily irradiated cells (80,000 r) on chloramphenicol agar increases the capacity of *E. coli* for phage T 3.

It is quite possible that this favourable effect is due to the fact that chloramphenicol inhibits protein synthesis, and as the polymerization of nucleic acids is also inhibited, a certain amount of DNA precursors are accumulated, which can be used for restoration.

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#### DISCUSSION

MARCOVICH: There was an experiment in which chloramphenicol was added before the release of phage. How could it exert any influence, if the phage particles were already completely formed?

HERČÍK: Chloramphenicol was added at the end of the latent period.

MARCOVICH: What is the explanation for the results obtained?

HERČÍK: It is very difficult to explain, since it should be borne in mind, that the quantity of the phage is very small—only about two or three phages are released in the *Escherichia coli* suspension. Because of this, two possibilities present themselves: the first one is that phages are released by one or two surviving cells, since there are always surviving cells; the second possibility is that phages are released at a very slow speed by several hundred cells. I cannot say with certainty which of these two possibilities is more probable.

POLLARD: When you changed over to agar with chloramphenicol, what was the medium—the same as in the case of the suspension?

HERČÍK: Yes, the same.

MARCOVICH: Did you allow the bacteria to grow before seeding with the phage? Under these conditions the quantity of the phages formed would have had no influence.

HERČÍK: It would depend upon the number of bacteria which had been damaged by the irradiation.

# THE ACTION OF IONIZING RADIATION ON THE CELLULAR SYNTHESIS OF PROTEIN†

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## SUMMARY

The experiments described are all in accord with the idea that the immediate synthesis of protein is caused by organelles which are sensitive to ionizing radiation in the manner expected for ribosomes *in an extended form*. The volume of the sensitive regions agrees with the volume of an 80S ribosome and this, independently, substantiates the work of McQuillen, Roberts and Britten (1959) who have shown that the short term incorporation of  $^{35}\text{SO}_4$  is into 70S ribosomes.

The long term effects of radiation are much more difficult to interpret. In separate experiments we have found that there is a reduction in the rate of increase of DNA in irradiated cells. Possibly this means that there is some kind of disruption of the bacterial nucleus which produces an unbalance in the cell and reduces cellular synthetic action later on. Partially successful attempts to express this theoretically have been made (Pollard, 1960), but it cannot be claimed that the process is fully understood.

Synthesis of protein is an essential characteristic of every living system. It is now known that it takes place in several stages and that different mechanisms are involved with each. The present studies were made with the aim of using the disruptive action of ionizing radiation to give some information on the nature of the process of protein synthesis. The work reported is not complete: it is still in progress. Nevertheless some definite facts have been established and some conclusions can be drawn regarding protein synthesis, and also on the sensitivity of some parts of the cell to ionizing radiation.

The cell employed throughout the work is the bacterium *Escherichia coli*.

## PHYSICAL ACTION OF IONIZING RADIATION

The pioneer work of Lea and others in the pre-war years has been greatly extended in the past ten years in the Yale Biophysics Department. The work has been reported in various review articles (Pollard,

† The work reported here was largely supported by the U.S. Atomic Energy Commission.

‡ For 1960-61 visiting Professor at the Pennsylvania State University.

Guild, Hutchinson and Setlow, 1955; Pollard, 1959) and the important conclusions are as follows.

1. An ionization within the volume of a protein molecule or a nucleic acid molecule has a very high probability, in excess of 0.5, of removing its biological action. The mechanism of this action in protein is beginning to be understood and is probably related to the migration of excitation energy to an -S-S- linkage which thereby becomes sensitized. In nucleic acid it is probably breakage of the chain, or possibly cross-linkage.
2. A nucleoprotein molecule, such as a small virus, can also be inactivated by ionization. The probability of inactivation is less, but still high.
3. Estimates of the volume of biologically important molecules based on the theory that for activity to remain after irradiation they must have wholly escaped any ionization whatever, are very informative and have proved to be correct within a factor of two, with few exceptions.
4. Within cells, the radicals formed by ionizing radiation do not diffuse more than 30 Å before they encounter some structure or molecule which removes them.

The evidence in support of the above four conclusions cannot be given in this paper because time does not permit. Table I shows the results of irradiation of several enzymes, with the conclusions drawn from the statistics of complete escape. Table II shows some results of irradiating viruses. Table III shows the results of experiments largely by Hutchinson (1960) on which the distance of 30 Å has been estimated. It will be useful later to see what conclusions can be drawn about protein synthesis using the same analysis.

TABLE I

Material	Molecular weight deduced by radiation	Reported molecular weight
Penicillin	550	356
Catalase	110,000	250,000
Invertase	120,000	120,000
Pepsin	39,000	36,000
Chymotrypsin	50,000	23,000
Insulin	23,000	6,000
Trypsin	34,000	24,000
DNase	62,000	63,000
Alpha-amylase	145,000	100-200,000
$\beta$ -galactosidase	290,000	360,000

TABLE II

Virus	Diameter deduced from radiation (m $\mu$ )	Reported diameter (m $\mu$ )
Influenza A (infectivity)	42	80-90
Newcastle Disease (infectivity)	56	110
Shope papilloma (infectivity)	40	44
Measles	58	140
Southern bean mosaic virus	18	30

TABLE III

Enzyme	Cell	Diameter(m $\mu$ )		Diffusion distance in angstroms
		Dry	Wet	
Invertase	Yeast	6	12	29
Alcohol dehydrogenase	Yeast	1.3	28	31
Coenzyme A	Yeast	3	200	35
"	E.coli	15	200	17
$\beta$ -galactosidase	E.coli	9.6	11.4	9

## STATISTICAL ANALYSIS OF RADIATION DATA

If the idea of complete escape from ionization is to be used it is first necessary to determine the nature of the distribution of ionization. If the agent causing ionization is a fast electron the primary ionizations are widely separated along the track, which is itself very much subject to scattering. Thus there is every reason to treat the ionization produced by fast electrons, which includes the effects of gamma rays which generate fast electrons, as being spread statistically throughout any volume. The original tracks do not contribute regions of locally high ionization in any way that matters much. The ionizations themselves are not wholly understood for solid or liquid material. If we apply the results found for gasses to more dense material then the average energy release at a primary ionization is 100 eV. There is a wide distribution of values around this average, but it can be used as a basis for statistical reasoning. We suppose that all the secondary ionizations consequent on the primary process occur within a distance of a few angstrom units from the primary ionization. This will not be true for the more energetic secondary electrons, but it is true for those near to the average. It is not a bad first approximation. Using this value, we take the energy lost per cubic centimeter in the material bombarded as a result of the radiation, express it in electron volts and divide by 100. This then gives  $I$ , the number of clusters of ionization per unit volume.



If the sensitive region which we are interested in has a volume  $V$  cubic centimeters, then the average number of clusters of ionization occurring within  $V$  is  $IV$ . We are interested in the probability of complete escape. This can be estimated from the Poisson formula, from which we deduce that if  $IV$  is the average number of "hits" then  $P(0)$  the probability of no hit at all is

$$P(0) = e^{-IV}.$$

If we state that the ratio of activity remaining,  $n$ , to that initially present,  $n_0$ , is a measure of  $P(0)$  we deduce that

$$\frac{n}{n_0} = P(0) = e^{-IV}$$

or

$$\ln \left( \frac{n}{n_0} \right) = -IV \quad (1)$$

In many cases it is found that the activity remaining is related to the dose by the relation

$$\ln \left( \frac{n}{n_0} \right) = -(\text{constant}) (\text{Dose})$$

This can be converted into the same form as equation (1) by calculating  $I$  from the dose. The constant is then immediately expressible as  $V$ . The volume  $V$  is informative in regard to the nature of the biological unit responsible for the activity which is lost because of radiation.

#### STATISTICS OF HEAVY PARTICLE RADIATION

If irradiation by heavy particles is used then the ionization cannot be considered to be randomly distributed in volume, as the above reasoning cannot be used. The heavy particles are much more nearly *line probes* which are randomly distributed in *area*. Unfortunately this is only approximately true, because the secondary electrons along the path of the particle spread ionization away from the track. The amount of this spread can be estimated and it is possible to use heavy particle radiation to make estimates of the *area* of a molecule or molecular system, and of its *thickness*. Neither are to be considered as precise, yet it is clearly possible to tell whether an organelle is long and thin, or has one dimension small, or whether it is more nearly spherical, with no thin dimension.

When such irradiations are performed, then, the dose is expressed as particles per square centimetre,  $D$ , and if the effective cross-section is  $S$  an area which is now the equivalent of the volume,  $V$ , used formerly,

we have, as before

$$\ln \left( \frac{n}{n_0} \right) = -SD \quad (2)$$

We first correct  $S$  for the secondary radiation off the path, using the method given by Pollard and Barrett (1959) and then, if  $t$  is the effective thickness and  $i$  the number of primary ionizations per cm path of the particles,

$$S = S_0 (1 - e^{-it}) \quad (3)$$

With sufficient variety in the linear energy transfer, which determines  $i$ , we can estimate both  $S_0$  and  $t$ . These figures are then useful to examine the character of the cell organelle responsible for the effect observed.

#### RADIATION ACTION ON AMINO ACID UPTAKE

Bacterial cells were grown on minimal medium, using phosphate for buffer, and 5 grams of glucose per litre. At a concentration of  $4 \times 10^8$  cells per ml samples were taken and irradiated either in a cobalt source, or in a cyclotron. For cobalt radiation they were irradiated in screw-top culture tubes. Some radiation was done in the frozen state at  $-80^\circ\text{C}$ . For cyclotron radiation the cells were placed on fine grain filters backed by a porous layer soaked in minimal medium. The temperature during radiation was  $2^\circ\text{C}$ .

After radiation the samples, including unirradiated controls, were incubated in minimal medium with addition of the particular labelled substance under study. At two minute intervals 2 ml samples were withdrawn and either filtered at once on a bacterial filter (collodion membrane, average pore size  $0.85 \mu$ ), constituting the "whole cell" fraction, or allowed to stand in cold trichloroacetic acid (TCA) for an hour at  $2^\circ\text{C}$  before filtering. The whole cell fraction was washed with minimal medium, the TCA insoluble fraction with 5 per cent TCA. The filters were then dried and counted under a thin window counter.

The results for one case, arginine, are shown in Fig. 1. The five graphs show the uptake in the whole cell (upper graph) and TCA insoluble fractions. It can be seen that the normal behaviour is a rapid uptake in which all the radioactivity is rather quickly incorporated. The difference between the whole cell and TCA insoluble fraction, designated as "pool", is small. This is in agreement with the findings of Roberts *et al.* (1957). For small doses of radiation there is no readily detectable effect at all. At 192,000 r the whole cell uptake rises nearly normally and then falls, probably because the cell membrane develops

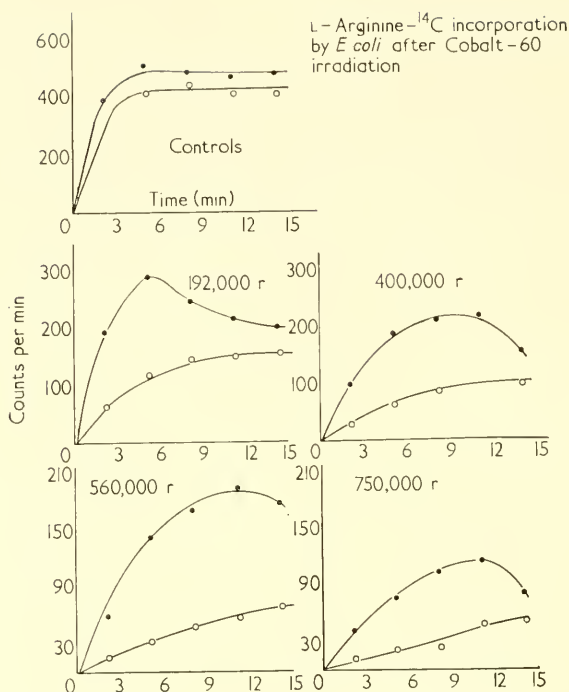


Fig. 1.—Incorporation of L-arginine into the whole cell (upper) and TCA insoluble fraction as affected by various doses of <sup>60</sup>Co  $\gamma$ -radiation. There is always a steady increase in the TCA insoluble fraction, but the whole cell fraction rises and falls.

leakage due to faulty growth. The fall is not observed in the same way for all amino acids. The uptake into the TCA insoluble fraction is reduced and also delayed in its rapidity of uptake. Steadily increasing the dose steadily increases all the effects. The doses used, it must be noted, are very large.

In Fig. 2 the amount of uptake at 6 min is plotted against dose using a logarithmic scale for the uptake. It can be seen that the relationship obeyed is in accord with the requirements of equation (1) and so a value for the inactivation volume,  $V$ , can be deduced.

In Fig. 3 is shown the effect of deuteron bombardment. The control behaviour is slightly modified by the fact that the cells are on a membrane and have to be removed before incubation, but the effect of radiation is clearly the same. Again, the doses used are very large.

This same procedure was followed for histidine, leucine, isoleucine, proline and methionine. In addition, the uptake of <sup>14</sup>C-uracil and <sup>14</sup>C-glucose was studied.

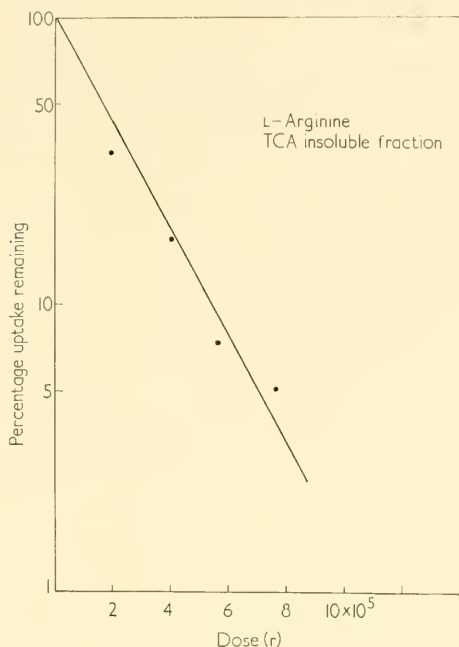


FIG. 2.—The percentage uptake of L-arginine at 6 min as a function of dose. The plot of the percentage is on a logarithmic scale and it can be seen that if  $n/n_0$  is the ratio of uptake to original uptake, then the relation  $\ln(n/n_0) = \text{constant} \times \text{dose}$  is obeyed.

The results are shown in Fig. 4. In Fig. 4 we plot the uncorrected cross-section found from the relation  $\ln(n/n_0) = -SD$  against the number of primary ionizations per cm generated by the bombarding particle. We can include the data for cobalt bombardment by realizing that if  $i$  is the number of primary ionizations per cm per heavy particle then for particles of very low linear energy transfer the equivalent volume ionization density is the number of particles per  $\text{cm}^2$  ( $D$ ) times the ionization per cm for each one ( $i$ ) so that  $I = Di$ . Since equations (1) and (2) are equivalent we can set  $VI = SD$  or  $VDi = SD$  or  $V = S/i$ . Thus  $V$  appears as an initial slope, on the  $S$  versus  $i$  graph and has been so represented. It can be seen from Fig. 4 that uracil and glucose behave quite differently toward heavy particle bombardment than the other metabolites, with the possible exception of methionine. The sensitivity to cobalt irradiation is quite high and yet the expected high cross-section does not develop for heavy particle bombardment. The sensitive region is roughly spherical of radius 160 Å in the case of uracil and 90 Å for glucose. Since some radical migration must be occurring it would be expected that the actual organelle size is rather less. Both

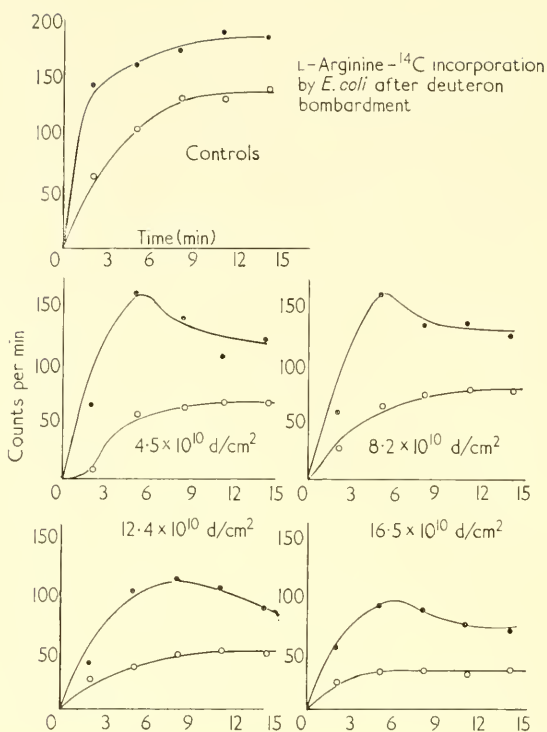


Fig. 3.—Incorporation of L-arginine as affected by various deuteron bombardments.

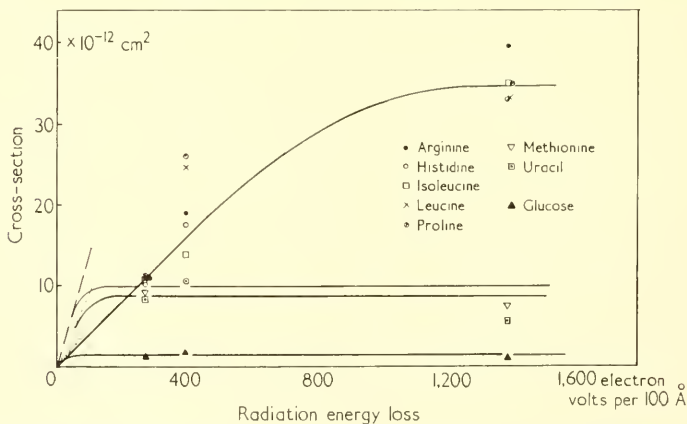


Fig. 4.—A plot of the sensitive cross-section for seven metabolites against the rate of energy loss. The initial slope is found from  $^{60}\text{Co}$  inactivation. Three groupings appear: arginine, histidine, leucine, isoleucine and proline are all characterized by high sensitivity at high rates of energy loss; methionine and uracil have rather low sensitivities for such radiation, and glucose is consistently low. Probably metabolites of the first grouping involve long thin objects, but the others are more nearly spherical.



these could be represented by one or other of the various classes of ribosome. It is possible that methionine also fits into this class. In any event, the sensitivity of methionine uptake markedly differs from the other amino acids, suggesting that some other mechanism is involved for the incorporation of methionine.

The data for the other 5 amino acids agree with a sensitive region having a molecular weight of between  $3.5$  and  $5 \times 10^6$  and having one thin dimension, roughly estimated as  $30 \text{ \AA}$  thickness. The molecular weight agrees rather well with that of the ribosomes, being equivalent to a particle of 70 Svedberg units. The shape does not agree and it is suggested that for these 5 amino acids the operating condition is one which is unfolded, while in preparations in broken cells, the particle is rolled up or folded in some way. Perhaps it is the function of the energy source to provide the unfolding. This work was done in collaboration with Dr. E. S. Kempner.

#### RADIATION ACTION ON THE FORMATION OF AN ENZYME

The previous study is concerned with the almost immediate incorporation of a labelled amino acid in the presence of a minimal medium. The radioactive tracer method is very sensitive and the studies are

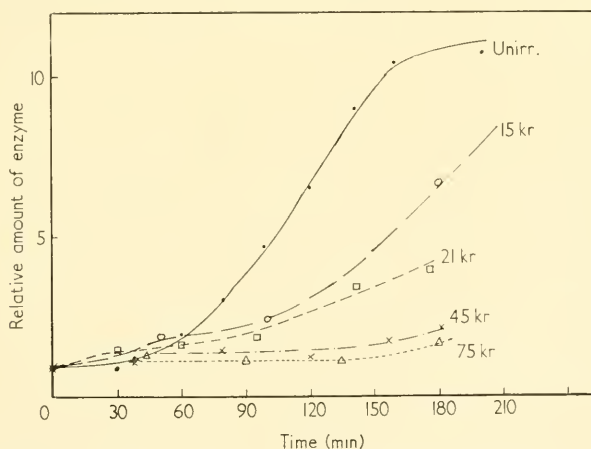


Fig. 5.—The effect of various doses on the time course of production of  $\beta$ -galactosidase in cells already induced. For low doses there is a small increase initially. In general the enzyme is depressed and delayed.

relatively simple. If we turn to the formation of an actual enzyme,  $\beta$ -galactosidase, the sensitivity of assay is harder and it is not so easy to detect differences at short times. Using cells already induced by growth

on lactose, no effect of radiation up to 75,000 r could be seen up to 45 min after irradiation. To make the enzyme assay it is necessary to open the cells. The method used was rapid expansion under pressure and while the recovery of enzyme was always good the data scatter more than for the radioactive uptake experiment. Nevertheless there is no reason to suppose that the immediate effect of ionizing radiation on enzyme formation differs greatly in sensitivity from that observed for amino acid incorporation. At later times after irradiation the story is quite different. A dose as low as 15,000 r quite clearly gives a reduced yield of enzyme. Data for different doses and different times are shown in Fig. 5. It is quite clear that a process which involves the development of the cell is at work. A clear decision as to the nature of this process cannot, at the moment, be made.

#### RADIATION ACTION ON THE PROCESS OF INDUCTION

For this particular enzyme it is necessary that cells which have grown on glucose become adapted, or induced, before they are able to make the enzyme if they are grown on lactose. The above described procedure can be applied to cells which have not been induced and radiation action on the process of induction can be studied. A typical time sequence is shown in Fig. 6. The first clear reading of the enzyme

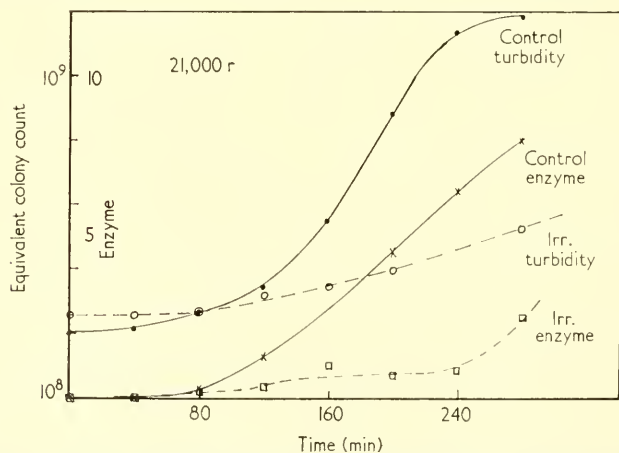


Fig. 6.—The effect of 21,000 r on the process of induction of  $\beta$ -galactosidase. The effect on turbidity is shown for comparison.

in the control appeared at about 60 min and thereafter rose sharply and steadily. The irradiated cells also showed a small reading at 80 min which corresponds to the same as the control, but thereafter rose much

less definitely and appeared to flatten somewhat between 140 and 240 min, even though the culture was showing an increased turbidity. After 4 hr there is an increase in enzyme, but we also noticed some enzyme leaking from the irradiated cells into the medium, a feature not observed in the controls.

Increasing the dose had the effect of depressing the development of enzyme into the region hard to measure. After 320 min, a culture which had received 60,000 r barely gave a readable amount, although small readings had been recorded when the control began to show the presence of enzyme. The data are summarized to some extent in Table IV. The ratio of enzyme for irradiated cells to control cells is shown for various times. It was also observed that the amount of enzyme, for both control and irradiated cells approximately follows the development of turbidity.

It is once again clear that a process involving cellular development is involved.

TABLE IV

Dose (r)	Ratio of enzyme to control at intervals after induction			
	0 min	30 min	90 min	180 min
21,000	1	0.5	0.26	0.13
30,000	1	0.5	0.4	
45,000	1	0.7	0.5	0.04
60,000	1	0.3	0.12	0.06

UPTAKE OF  $^{35}\text{S}$  AS SULPHATE

Radioactive sulphate is primarily incorporated as protein. A general idea of protein synthesis can be therefore obtained from an observation of sulphate uptake after irradiation. It was found that very little effect was observed on the ability of the cell to incorporate sulphate for times up to 12 min unless doses over 200,000 r were used. A rough estimate of the 37 per cent survival dose is 300,000 r. The immediate effect of radiation is thus very much like the effect on the uptake of amino acids and so is probably due to the disruption of ribosomes in some way. If longer times of uptake are considered there is a marked effect as can be seen from Fig. 7. The uptake follows the normal and then, rather sharply, deviates from the exponential line and becomes linear, increasing at lower rates with higher dose. The appearance is rather like the production of enzyme and confirms the general behaviour seen there. Some secondary development process is again taking place. The irradiations described were under conditions which were nearly anaerobic. If oxygen was bubbled, a given dose had definitely more effect, both in

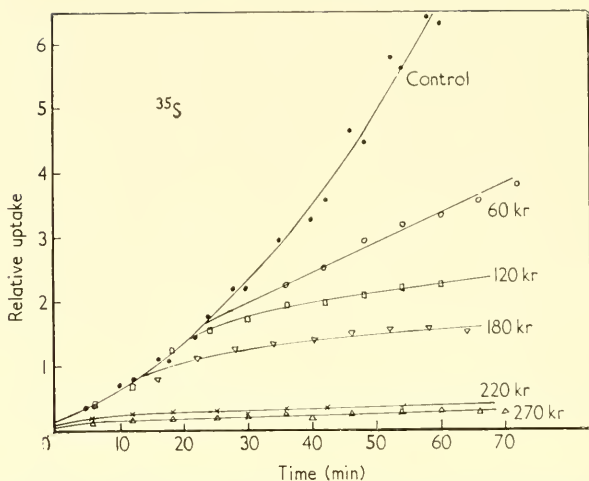


Fig. 7.— The uptake of  $^{35}\text{S}$  after various doses. The linear uptake can be seen, also the fact that for very high doses there is an initial effect.

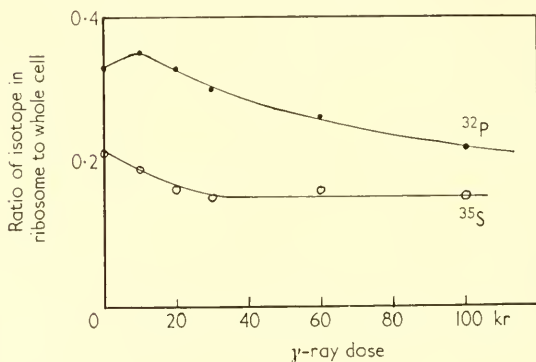


Fig. 8.—The radioactivity observed in the ribosome fraction as a function of dose. The relative amount of  $^{32}\text{P}$  rises slightly and then falls.

the stage of departure from exponential incorporation, and also in the reduced slope.

#### INCORPORATION INTO RIBOSOMES

In order to attempt to see what mechanism in the cell is damaged in such a way as to cause the delayed effects the incorporation of  $^{32}\text{P}$  and  $^{35}\text{S}$  into ribosomes was measured. If cells are broken open in the presence of sufficient concentration of  $\text{Mg}^{++}$  the ribosomes remain intact and can be separated by differential centrifugation. After clearing

cell debris, the pellet formed after three hours' centrifugation at 120,000 g was taken to be ribosomes, following the work of Roberts *et al.* (1958). The amount of  $^{32}\text{P}$  and  $^{35}\text{S}$  in the ribosomes of irradiated cells was compared to that in normal cells, in both cases after incubation for 60 min with labelled  $\text{PO}_4$  and  $\text{SO}_4$ . The results obtained, in work in collaboration with R. Wax, are shown in Fig. 8. In the case of  $^{32}\text{P}$  there seems to be a very slight increase in the incorporation of  $^{32}\text{P}$  into the ribosomes for small doses. For higher doses the proportion falls, with a sensitivity which corresponds to that found for the uptake of amino acids. In the case of  $^{35}\text{S}$  there is a fall to a constant level.

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### DISCUSSION

HERČÍK: Is there really a bend in the first curve?

POLLARD: It is possible that it may depend on the precision of measurements and at present we should be cautious about it. We have as yet but few data pointing to the presence of a bend.

MARCOVICH: Is there a formation of the inductive enzyme within the cell or does an inhibition of the enzymatic activity take place?

POLLARD: This is not yet clear. It would be interesting to find such an inducing agent which would not itself be a metabolite.

ALEXANDER: While calculating the sizes of the protein particles in complex systems did you take into consideration the possibility of energy transfer from one molecule to another?

POLLARD: Experiments carried out on pure substances and on non-purified preparations (for example on pure DNA and on DNA in yeast) have shown that the accompanying substances do not alter the effect considerably, only about twofold.

ALEXANDER: Protection by transfer of energy to other substances may alter the effect not twofold, but up to tenfold.



POLLARD: It seems to me that within the cells there is no considerable protective effect.

ERRERA: Did the cross-section of the protein synthesizing particles for the  $^{35}\text{S}$  incorporation coincide with that for the methionine? Were the cross-sections compared for the cells with deficient and adequate amino acid and nucleotide supply?

POLLARD: The first question we may answer in the affirmative, although it would be interesting to determine the specificity of the methionine's behaviour compared with that of its homologue. The second problem we have not yet studied although we intended to in order to determine whether ribosomes can exist in the extended as well as in the contracted form.

PASSYNSKY: In the basic equations  $\ln(n/n_0) = -V$  and  $\ln(n/n_0) = SD$  the values  $V$  and  $S$  are not the volume and real surface of the target in the strict meaning of the word. They are but factors of probability, possessing the dimensions of volume and surface, but they differ from these by dissimulated non-denominated coefficients of proportionality  $K$ . If  $K = 1$ , then these concepts coincide. For pure proteins results may be compared with those obtained by other methods and it could often be assumed that  $K = 1$ ; this approach then gives some interesting results. But with regard to such complex intracellular systems as systems of protein biosynthesis or amino acid incorporation we cannot be sure that the condition  $K = 1$  always holds true, and in such cases the dimensions calculated may differ considerably from the real values.

POLLARD: That is quite correct. In model experiments on simple substances the agreement was good enough. Unfortunately, for the study of protein synthesis in a living cell there are no other approaches available to evaluate the dimensions of the structures involved.

We believe that our investigations contribute some information and deserve some consideration along with other proofs. It is possible that they have some importance.

BACQ: It is possible that the peculiar behaviour of methionine is accounted for by its utilization by the cell not only for protein synthesis but for other functions, for example, connected with methyl group transfer.

POLLARD: This possibility exists for other amino acids too.

TOBIAS: Irradiation with doses as large as  $2 \times 10^5$  r could produce membrane lesions and loss of a quantity of certain substances (ions, nucleotides) from the cell which could alter the calculated results.

POLLARD: Within 15 minutes we have not seen any loss of substances with the exception of arginine. Since there is an increase in cell mass we believe that active protein biosynthesis was taking place.

TUMERMAN: Are there data available on the use of the electron microscope for the direct measurement of the areas studied, since their dimensions are within the resolution range of this method.

POLLARD: While studying irradiated viruses and ribosomes of the irradiated cells with the electron microscope, no difference was found compared with non-irradiated preparations. It is unlikely that this approach would allow us to discern differences in the process of amino acid incorporation.

EIDUS: Is it possible to relate the values for the diffusion tracks in dry objects given in the report to definite radicals (for example, those produced during radiolysis of the residual water) or to migration phenomena recorded by the ESR method?

POLLARD: We have not studied the detailed picture of the process but have just tried to observe effects on biological action.



# CHEMICAL SPECIES INDUCED BY X-RAYS IN CELLS AND THEIR ROLE IN RADIATION INJURY†

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## SUMMARY

The experimental evidence for the existence of several chemical species in the dry bacterial spore after X-irradiation is reviewed and examined critically. One very short-lived species is recognized only if oxygen is present during the time of irradiation; another becomes toxic anoxically and is recognized only if hydrogen sulphide is present during irradiation. Certain characteristics of the long-lived free radicals that become toxic to the cell if they combine with oxygen are examined with special attention to reconciliation of biological and chemical evidence with physical evidence gained from ESR techniques. Post-irradiation thermal annealment and post-irradiation treatment with nitric oxide reduce the biological effectiveness of X-rays to the same degree; but the former approximately halves ESR signals, whereas the latter removes all signals almost completely. One interpretation of these results is that two general kinds of radicals are formed, one of which is thermally annealable and biologically important the other of which is not. Both react with oxygen and with NO. While the removal of the two kinds of radicals with NO results in obliteration of the ESR signal, the biological result is the same as that seen after thermal annealment because only those radicals are important biologically. Another interpretation is that reaction of one kind of radical with NO results in a harmless complex, whereas reaction with a second kind results in a harmful complex. These studies and others similar to them, especially those involving the role of water in these effects, should lead eventually to some understanding of the early effects of high energy radiations in cells.

## INTRODUCTION

The "initial" effects of high energy radiations in living systems depend upon the same parameters as those in non-living ones, and any one interested in understanding the biological effects of irradiation should find assistance from studies on the interactions between radiations and purely physical systems. For instance the relationship between stopping power and atomic number is undoubtedly the same in protoplasm as it is in a plastic, the very early chemical changes (i.e. those strictly non-enzymatic in nature) should not be different, and the

† This work was performed under the auspices of the United States Atomic Energy Commission.

fates of the new chemical species must be governed by the same physical laws.

In order to apply the knowledge of the physical systems to the biological, one must design experiments to test parameters of the types utilized in ordinary physical and chemical radiation experiments. It is clear, however, that most functioning biological systems operate successfully only within narrow environmental limits, and it is difficult to apply many physical experimental techniques to them. In consequence there has been little progress in the understanding of early events induced by high energy radiation in living systems.

Very recent studies of two general kinds provide hope that descriptions will be forthcoming of some of the early radiation-induced events in cells that are important in bringing about biological effects. In aqueous systems the use of very short pulses of radiation coupled with fast, sensitive, detecting devices can circumvent the severe difficulties one meets in analyzing a series of events that goes to completion in a fraction of a second (see L. H. Gray in this Symposium). The other general approach that promises early success is the use of dry biological materials that can be exposed to a wide range of environmental circumstances like those useful in physical experiments, and in which, in addition, reactions are sufficiently slow to allow analysis with ordinary techniques. Direct extension of the results obtained with this kind of material to the wet biological system is difficult, because water undoubtedly modulates the series of reactions. But the evidence from the dry system will provide a basis for asking many questions, one of which is the difference made by the introduction of water.

The second approach is being pursued in several laboratories in which a variety of biological materials such as plant seeds, pollen, dry bacterial cells and spores, and viruses are being investigated.

In this laboratory we have been systematically investigating the response of dry bacterial spores to X-rays under a variety of environmental circumstances. We have evidence for several of the early events, and have been able to describe the characteristics of some of the products of irradiation and their relationship to radiation-induced damage. In this paper we shall summarize briefly the evidence for several kinds of identifiable chemical species, for the relationship of oxygen to them, and the extent to which they participate in the biological damage caused by X-rays. The results demonstrate the existence of the following: an oxygen-independent portion (Class I) that can be sub-divided into a radical species (Ib) and one that may not be radical-like (Ia); an oxygen-dependent portion that consists of very short-lived species



(Class II); and an oxygen-dependent portion that consists of long-lived free radicals (Class III).

### THE BACTERIAL SPORE SYSTEM

We use spores of a strain (ATTC #8245) of *Bacillus megaterium* that has demonstrated good sporulating capacity. The spores are mounted in known numbers on precipitated cellulose discs (called the Millipore Filter) 0.1 mm thick (Powers *et al.* 1957; Kaleta and Powers, 1958). The discs carrying the spores are dried in a chamber at less than 1 mm Hg. pressure over a drying agent for several hours, and are kept in this chamber until ready for use. Colony formation is induced by putting the paper disc on an absorbent pad saturated with liquid nutrient medium. The spores germinate and give rise to colonies on the surface of the disc. Control, unirradiated discs demonstrate 100 per cent recovery of mounted spores.

### Experimental methods

When exposing the spores to X-rays, we utilize the chamber shown in Fig. 1. (Webb *et al.*, 1958). The discs are put on the bottom of a stainless steel cylinder. The gas surrounding them can be controlled by

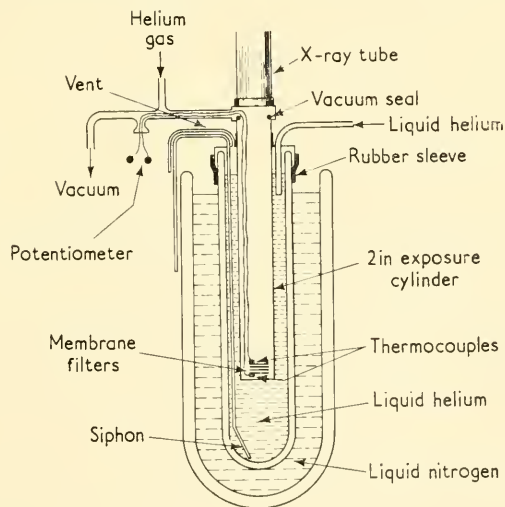


FIG. 1.—Diagram of the basic exposure chamber, for controlling atmosphere and temperature before, during, and after irradiation. (From Webb *et al.*, 1958).

means of pumps and valves connected to the manifold that is between the cylinder and the X-ray tube. The temperature within the cylinder

can be maintained by means of constant-boiling liquids in the surrounding vessels. The temperatures during irradiation are monitored by means of thermocouples placed at the bottom of the cylinder.

The measure of radiation sensitivity in all the studies described here is the slope of the survival curve expressed in reciprocal kiloroentgens. The response curves usually have small shoulders in the low-dose regions. A convenient expression for describing them is

$$\text{Fraction surviving} = 1 - (1 - e^{-kD})^n$$

in which the two constants have the following meanings:  $k$  is the slope of the curve in  $\text{kr}^{-1}$  and is the measure we use of radiation sensitivity;  $n$  is a measure of the size of the shoulder in the particular experiment, and in some other papers is referred to as the "hit number". We refer to the slope as the "inactivation constant", and to  $n$  as the intercept number, because of the fact that extrapolation of the straight line portion of the response curve on a semilog plot gives the value of  $n$  as the intercept on the  $y$  axis. All of the experimental variables are tested with complete survival curves, and all response curves are reduced to their respective slopes. The values of  $n$  in these experiments vary about a mean of 1.30. They are regarded as constant and without significance in the studies we review in this paper. The biological response being measured in these experiments is the ability of the irradiated spores to germinate and to produce visible colonies; no other endpoint is being considered.

### THE LONG-LIVED RADICALS (CLASS III)

The radiation response can be divided into a number of categories, that is, the individual cell may be damaged in a number of ways. The first of these that we shall consider is that damage which is brought about as the consequence of the production of chemical species with unpaired electrons, termed "free radicals". In our system these have appreciably long lifetimes, and are related to oxygen in a particular way in the production of the radiation damage.

#### *Thermal evidence*

We repeated with the spores the study of the basic temperature response of radiation sensitivity in an oxygen-free environment studied earlier in T 1 virus (Bachofer *et al.*, 1953), and noted that, as the temperature is varied from very low temperature (5°K) to higher temperatures, radiation sensitivity does not change until the temperature reaches 125°K (Webb *et al.*, 1958; Webb and Powers, 1961). At that

point, radiation sensitivity increases slowly to a maximum at 30°C (303°K) (Fig. 2). It then decreases markedly, and reaches a minimum at 80°C at a level that is appreciably below that observed even at the very lowest temperatures. While it is not possible to interpret these results in terms of free radicals from this evidence alone, we shall see that this is consistent with and contributes to, the free-radical hypothesis. The marked inversion of radiation sensitivity at the higher temperatures is the consequence of annealing of free radicals. The effect of post-irradiation exposure of the spores to heat is described in the next paragraph.

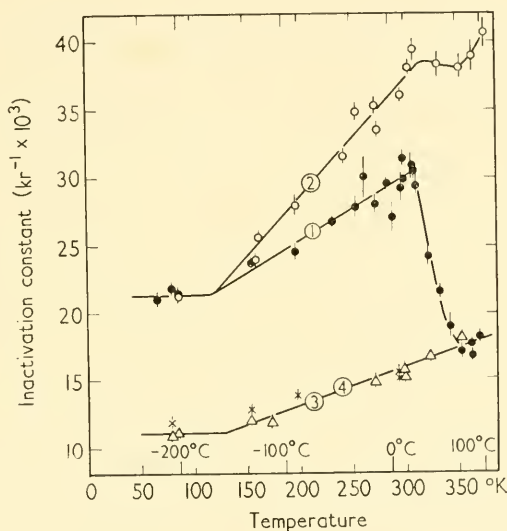


FIG. 2.—The relationships among radiation sensitivity of spores (the ordinate), temperature during irradiation (the abscissa), and post-irradiation thermal and NO treatments. (From Powers *et al.*, 1960a).

H <sub>2</sub> O-free Spores		
Curve	Exposure gas	Post-irradiation treatment
●	N <sub>2</sub> , He	—
○	O <sub>2</sub>	—
Δ	N <sub>2</sub> , He	80°C
×	N <sub>2</sub> , He	NO

### Nitric oxide

In another series of experiments, we used the gas nitric oxide as a modifying agent (Powers *et al.*, 1960b). This gas is most effective when

presented to the spore after irradiation. In Fig. 2 we show that approximately 50 per cent of the total effect of irradiation can be removed by post-radiation treatment of spores with nitric oxide. This effect is independent of the temperature during irradiation, and the effect of exposing the spores to X-rays over the temperature range and treating them with nitric oxide after irradiation is to shift the entire curve down without changing its general form. The same figure demonstrates that coincident values are obtained by post-irradiation heating of the irradiated spores for 15 minutes at 80°C.

Heat and nitric oxide exposure after irradiation accomplish the same thing; namely, they induce changes in the irradiated spore that prevent the development of part of the damage expected from the given radiation dose. The coincidence of these results, the well-known action of nitric oxide in reducing free radical concentrations in physical systems, and the well-known effect of heat in reducing free radical concentrations, lead us to conclude that the portion of radiation-induced damage that is not observed following these treatments is caused by radiation-induced free radicals that have appreciably long lives. It should be noted that this radical component is independent of the temperature (below 30°C) at which the spores are irradiated—the overall relationship between temperature and radiation sensitivity is the same in the restored and in the unrestored spores.

When nitric oxide is present during irradiation, the degree of protection observed is less than that seen when nitric oxide is given after (Powers *et al.*, 1960b). In Fig. 3 we see that the level (inactivation constant) to which nitric oxide protects the cells when present during irradiation is approximately 25 per cent higher than that seen when nitric oxide is given after irradiation. This result is understandable if we postulate two actions of nitric oxide: one, a protective action that is due to the scavenging of radicals by nitric oxide that prevents their becoming toxic to the cell; and the other an enhancing effect of nitric oxide on the action of X-rays. The enhancing effect is much smaller than the protective effect, and the net result of the two actions is a lower protective action of nitric oxide when present during irradiation compared to its action afterwards.

### *Hydrogen sulphide*

Because of the interest in protective chemicals containing sulphydryl groups, and because of the possibility that these may act by scavenging radicals, we have tested a number of sulphydryls for their action against the long-lived radical component. Using the gas hydrogen sulphide (Powers and Kaleta, 1960), we have been able to show that

when given after irradiation it is equivalent to nitric oxide given after irradiation, and to post-irradiation heat. The level to which the cells are protected by the gas is approximately the same as that to which radiation sensitivity is reduced by heat and NO. This result is consistent with the interpretation that this part of the effect is due to long-lived free radicals: in this instance  $\text{H}_2\text{S}$  is donating a hydrogen atom to the free radicals, repairing these before they can become damaging.

When hydrogen sulphide is present at the time of irradiation, a degree of protection is seen exceeding any that we have observed in any other

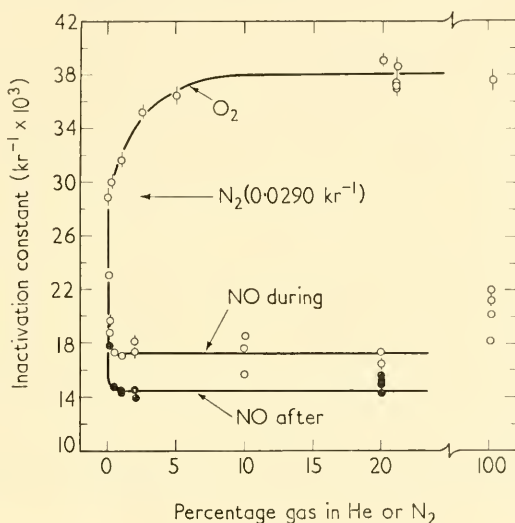


Fig. 3.—Changes in radiation sensitivity with concentration of  $\text{O}_2$  and NO present at the time of irradiation. (From Powers *et al.*, 1960b).

circumstance. This indicates the presence of another kind of radical that can accept hydrogen atoms from hydrogen sulphide. The lifetime of this species must be very short compared with the lifetime of the radicals that can be removed by post-radiation treatment. This is discussed below.

### *The role of oxygen*

The relationship of oxygen to these effects is revealed by the following series of experiments. When we irradiate spores in the presence of oxygen, we see a gradual increase in radiation sensitivity with an increase in oxygen concentration to a constant level that is reached at about 10 per cent oxygen (Powers *et al.*, 1960a, b and Fig. 3). The ratio



of the values at saturation is 1.25; that is, in the presence of oxygen, spores exhibit 35 per cent more radiation sensitivity than they do when irradiated in the presence of nitrogen and exposed to oxygen immediately after. This is a small oxygen effect compared with the oxygen effects of about 300 per cent usually observed in wet systems (Gray, 1957-8). This, however, is not the total oxygen effect that can be demonstrated in the bacterial spore. As shown in Fig. 2, the radiation sensitivity in the presence of oxygen increases more rapidly with increasing temperature than it does in its absence. At room temperature the ratio observed is about 1.25 as noted on the previous figure. Above 30°, the dramatic decrease in radiation sensitivity observed in the absence of oxygen cannot be demonstrated when oxygen is present (Powers *et al.*, 1959). This indicates that oxygen prevents the thermal reversion of the free radicals we observed in the other experiments. Verification of this can be obtained by irradiating the spores in nitrogen at any one of the temperatures below 30°, exposing them to oxygen briefly, removing the oxygen, and then treating with nitric oxide or heat. In these instances, the radiation sensitivity is not affected by heating or by the gas. Removal of the free radicals is possible, then, only if they are manipulated prior to their exposure to oxygen. These facts mean that the free radicals and oxygen form complexes that are irreversible, and that are damaging to the cell. This oxyradical or peroxyradical should be very strongly oxidizing, and should undergo a secondary reaction to produce damage to the cell. If the free radical is disposed of before oxygen is admitted, the damaging complex cannot be produced.

### *The physical evidence*

We have physical evidence in the form of electron spin paramagnetic resonance (ESR) analysis that supports our interpretation that part of the radiation damage is brought about by long-lived free radicals together with oxygen (Ehret *et al.*, 1960; Powers *et al.*, 1961). When the spores are irradiated at low temperature and an ESR spectrum is recorded, we observe a second derivative tracing with three peaks with 30 G spacings (Fig. 4). If these spores are brought to room temperature for brief periods, and then returned to low temperatures for reading, twin peaks 10 G on each side of the centre line grow in at the expense of the central peak. After about 20 minutes at room temperature, the fully developed spectrum can be seen: it consists of the originally observed triplet with a 1:2:1 configuration, and a newly developed doublet with a 1:1 configuration.

Therefore, the species produced immediately in the spore are not the only ones that are finally observed—migration of energy within the spore after irradiation must take place.

Also, the ESR apparatus reports to us only two general kinds of radicals, one an electron associated with two protons and the other associated with a single proton. The simplicity of this result is quite unusual, and probably indicates that in the well-ordered biological system, the energy from the X-rays, although deposited at random, migrates and becomes fixed in a limited number of places.

We have treated the irradiated spores by the methods that influence

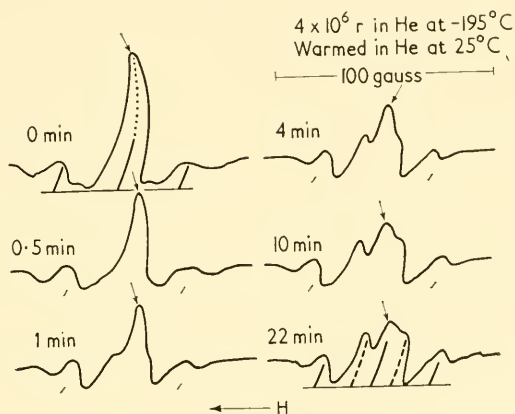


Fig. 4.—Changes in the ESR spectrum (2nd derivative) with time after irradiation of dry spores at 77° K. The spores were returned to 77° K for reading after each warming period. The arrow marks the position of  $g = 2.003$ . (From Ehret *et al.*, 1960).

radiation sensitivity as judged by biological criteria, and the results are shown in Fig. 5 (Ehret *et al.*, 1960; Powers *et al.*, 1961). When the irradiated spores are heated for 15 min at 80°, there is a loss in amplitude with no qualitative change in the signal. The decrease appears to be approximately 50 per cent, in agreement with the biological experiments. When the spores are exposed to oxygen, we see the gradual growth of a single oxyradical (peroxyradical) signal from the finely split signals of the two other radicals. The radicals become oxygen complexes; they seem to be irreversible, for removal of oxygen from the system does not affect the signal. This is expected according to the biological result. Furthermore, the oxygen signal that develops from the heated spores is small, in correspondence with the fewer number of radicals available to form the complex.

Now we discuss the question as to whether the radicals that cannot be annealed by heat are responsible for damage when they are finally

exposed to oxygen. When the irradiated spores are exposed to nitric oxide, almost complete obliteration of the radical signal is observed (Fig. 5). At first glance this seems to be anomalous, for the biological consequence of heat and the nitric oxide treatment is the same, whereas the physical result appears to be different. However, instead of indicating limited usefulness of the experimental approach, the result might be amenable to an interesting explanation.

Either of two possibilities exist. First, the compound formed by the reaction of oxygen and the radicals remaining after heat annealment

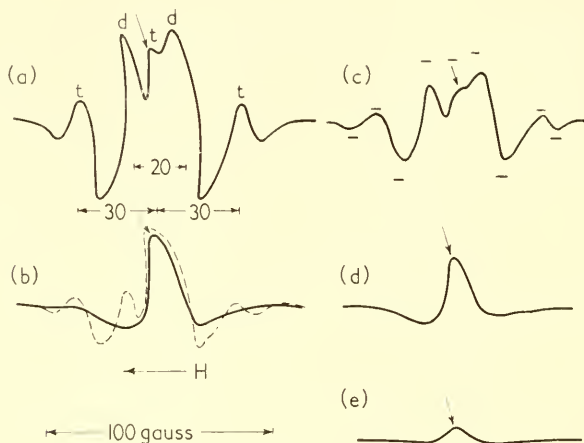


Fig. 5.—The effect of various treatments on the ESR signal seen in irradiated bacterial spores. (a) The fully developed doublet-triplet signal. (b) The signal observed after exposure of the irradiated spores to oxygen. The dotted line is the signal intermediate between (a) and the heavy line of (b). (c) The effect on (a) of heating the spores at 80°C for 15 minutes. (d) The effect of exposing the heated spores of (c) to oxygen. (e) The signal observed after the spores of (a) are exposed to NO. (From Ehret *et al.*, 1960).

may not be important in the damage that we see, for in the NO-treated cells these cannot be formed, yet the biological result is the same in the two instances. Or, second, reaction of nitric oxide with these radicals brings about cellular damage—that is, the reaction between nitric oxide and the residual, non-annealable radicals is equivalent to the reaction between these residual radicals and oxygen. The reason there is no evidence of the nitric oxide-radical complexes is that after reaction there are no unpaired electrons, but the chemical or biochemical consequences are the same as those seen following reaction with oxygen, forming compounds with unpaired electrons that can be measured by the ESR spectrometer. If this is true, we can subdivide the long-lived

radicals we observe once again into those which can be harmlessly removed by reacting with nitric oxide but are toxic after reaction with oxygen, and those which become toxic or damaged by virtue of their reaction with nitric oxide or with oxygen.

There are then two ways in which the subdivision of Class III into two components can be viewed, each implying different characteristics of the radicals in question. One is as follows:

### IIIa

- |                                     |  |
|-------------------------------------|--|
| 1. In presence of $\cdot\text{O}_2$ | $\text{R}_a\cdot \longrightarrow \text{R}_a\text{O}_2\cdot$ (toxic)        |
| 2. Removable by heat (Anneal.)      | $\text{R}_a\cdot \xrightarrow{\text{heat}} \text{R}_a\text{H}$ (not toxic) |
| 3. Reacts with $\text{NO}\cdot$     | $\text{R}_a\cdot \longrightarrow \text{R}_a\text{ON}$ (not toxic)          |

### IIIb

- |                              |   |
|------------------------------|---|
| Not annealable               | $\text{R}_b\cdot \longrightarrow \text{R}_b\text{O}_2\cdot$ (toxic) |
| Reacts with $\text{NO}\cdot$ | $\text{R}_b\cdot \longrightarrow \text{R}_b\text{ON}$ (toxic)       |

In this case the radicals  $\text{R}_b\cdot$  are not sufficiently mobile to be annealed by heat, or they are in some way shielded from  $\text{H}\cdot$  atoms, or some other characteristic prevents their annealment, so that they persist and become active  $\text{RO}_2\cdot$  or  $\text{RO}\cdot$  radicals when oxygen is admitted to the system. These very radicals are, however, available to  $\text{NO}\cdot$  as shown by the ESR data, and if the foregoing statement is true, i.e. if they do become toxic radicals even after high temperature treatment, then the combination  $\text{R}_b\text{ON}$  must be equally as toxic as  $\text{R}_b\text{O}_2\cdot$ . Heat treatment and  $\text{NO}$  treatment produce the equivalent results biologically when used alone, and no additive effects of sequential treatments of the two agents has been demonstrated.

If the above explanation is true, we see a distinction between the two radicals on two counts: one ( $\text{R}_a\cdot$ ) is heat annealable, and the other ( $\text{R}_b\cdot$ ) is not, both being toxic when in the form  $\text{RO}_2\cdot$ ; and one ( $\text{R}_b\cdot$ ) is toxic as  $\text{RON}$  and the other ( $\text{R}_a\cdot$ ) is not.

Another general possibility is seen by setting up the characteristics of the two radicals as follows:

### IIIa—All toxic when in $\text{R}_a\text{O}_2\cdot$ form

- |                                 |   |
|---------------------------------|---|
| 1. Annealable                   | $\text{R}_a\cdot \longrightarrow \text{R}_a\text{H}$ (not toxic)  |
| 2. Reacts with $\text{NO}\cdot$ | $\text{R}_a\cdot \longrightarrow \text{R}_a\text{ON}$ (not toxic) |

### IIIb—Not toxic when in $\text{R}_b\text{O}_2\cdot$ form

- |                                 |   |
|---------------------------------|---|
| 1. Not annealable, but          | $\text{R}_b\cdot \longrightarrow \text{R}_b\text{O}_2\cdot$ (not toxic) |
| 2. Reacts with $\text{NO}\cdot$ | $\text{R}_b\cdot \longrightarrow \text{R}_b\text{ON}$ (not toxic)       |

In this scheme, the differences between  $\text{R}_a\cdot$  and  $\text{R}_b\cdot$  is that one ( $\text{R}_a\cdot$ ) is annealable, the other ( $\text{R}_b\cdot$ ) is not; and only the annealable

radical forms the toxic  $\text{RO}_2^{\cdot}$  radical. The radical that is not effectively available for annealment apparently is not available for secondary reaction after formation of an  $\text{RO}_2^{\cdot}$  radical. It is trapped with respect to annealment, and, though available to  $\text{O}_2$ , as shown by the ESR result, it remains effectively trapped after reaction with  $\text{O}_2$ . Both are available to  $\text{NO}$ , and are converted into non-radical  $\text{RON}$  species, but since only one ( $\text{R}_a^{\cdot}$ ) is biologically important, the removal of  $\text{R}_b^{\cdot}$  by  $\text{NO}$ , an effect that results in an ESR spectrum different from that caused by annealment, does not cause reduction of damage beyond that observed after annealment.

A choice between these two general possibilities is not required by the evidence at hand today.

#### THE SHORT-LIVED, OXYGEN-DEPENDENT SPECIES (CLASS II)

Another class of radiation-induced chemical change can be inferred from the data previously examined. We have noted that  $\text{O}_2$  when present at the time of irradiation increases radiation sensitivity by 30 per cent. We have also seen that the presence of nitric oxide at the time of irradiation decreases the protective capacity of the gas by 20 per cent, as compared with nitric oxide given after irradiation; or, in other words, nitric oxide at the time of radiation increases radiation effect by 20 per cent, as compared with nitric oxide given after irradiation. It can be allowed that oxygen and nitric oxide accomplish the same thing in increasing radiation sensitivity. If oxygen and nitric oxide are in some way preserving energy within the cell for damage that is absorbed primarily by other molecules, then the lifetimes of these "excited" molecules must be very short. We are considering at the present time one possibility, namely, the action of nitric oxide and oxygen in catalyzing certain degradations of excited species (Powers *et al.*, 1960a). One of these could be the quenching of fluorescence, i.e. the non-radiative transformation of an excited species to some other form (e.g. an excited singlet to a triplet). In this case energy that in the absence of the two gases escapes from the cell in the form of a photon is preserved within the cell and becomes damaging when nitric oxide or oxygen is present.

#### THE SHORT-LIVED, OXYGEN-INDEPENDENT SPECIES (CLASS Ib)

One other class of radiation-induced chemical change can be postulated on the basis of the results of irradiating the spores in the presence of hydrogen sulphide. As noted earlier, radiation sensitivity appears to be below that observed when hydrogen sulphide treatment is given after irradiation (Powers and Kaleta, 1960). We conclude from this



that hydrogen sulphide can donate hydrogen atoms to a radical, or some species, that has a very short lifetime, and that can very rapidly become toxic to the cell in the absence of oxygen. This constitutes part of the oxygen-independent portion of the general response.

### THE RADIATION SENSITIVITY PROFILE

The studies described above enable us to construct a diagram interrelating the various kinds of damage we can infer from some of the experiments to date. We call this the "radiation sensitivity profile" (Fig. 6; Powers and Kaleta, 1960). The one we demonstrate in this

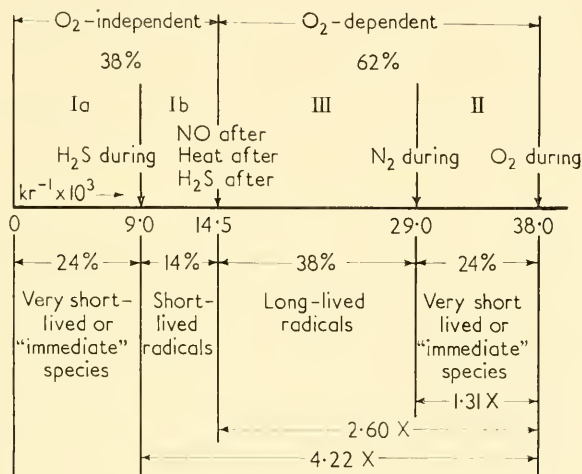


Fig. 6.—The radiation sensitivity profile of bacterial spores irradiated with soft X-rays (18 keV mean) at about 16 kr/min at room temperature. On the heavy horizontal line are indicated the inactivation constants observed under the described circumstances. The Roman numerals are the designations for the various components of radiation injury. (From Powers and Kaleta, 1960).

discussion is for X-rays of mean energy about 18 kV delivered at a dose rate of approximately 16,000 r/min at room temperature. It is necessary to specify these three items, since the relative response of each of the classes diagrammed may change with changes in these variables.

### COMMENTS

These and other experiments already performed or planned should result in a partial understanding of some of the early events in this cell caused by high-energy radiation. The experiments under way include the effect of dose-rate, linear energy transfer, and the interrelation of temperature with these. The very important problem of moisture is

being studied to attempt to bridge the gap between our dry system and the wet, metabolizing cell (Webb and Powers, 1961; Tallentire and Powers, 1961).<sup>†</sup> In this way we hope to make some contribution to the understanding of the interactions between high-energy radiation and functioning, living cells. Even at this time, we can describe a mechanism of action of sulphhydryl compounds in protecting the bacterial spore, and can wonder as to its applicability to other biological systems. In the case of the spore, the sulphhydryl ( $\text{H}_2\text{S}$  in this instance) protects by reducing  $\text{O}_2$  tension and by donating H atoms to free radicals making them harmless. The immediate  $\text{O}_2$  effect (Class II) cannot take place, and the long-lived radicals (Class III) are scavenged before  $\text{O}_2$  tension is increased again to the point that  $\text{O}_2$  can react with the  $\text{O}_2$ -dependent radicals. Also, the  $\text{O}_2$ -independent radicals of short lifetimes can be removed harmlessly as they are formed. Thus, the effect of  $\text{H}_2\text{S}$  is the equal of the total oxygen effect, and may exceed it somewhat.

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#### DISCUSSION

BACQ: At what dose-rate were the spores irradiated?

POWERS: In all the experiments reported here the dose-rate was 20,000 r/min. We considered it important that this condition be always fulfilled. It could be shown that as the dose-rate changes the general picture of the radiosensitivity is altered also.

<sup>†</sup> The work of Eidus and his colleagues (Eidus and Ganassi, 1959) is of interest in this connection. The loss of enzymatic activity of aqueous solutions (1 per cent) of mysoin after irradiation parallels our results on the bacterial spore. They observe an immediate oxygen effect, and a post-irradiation oxygen effect, that correspond closely in magnitude to these effects in the bacterial spore.

BACQ: At what time after the exposure to radiation were the spores treated with nitric oxide or hydrogen sulphide?

POWERS: We found that for 1.5 to 2 hr at room temperature the relative concentration of the radicals we dealt with did not change considerably. This point was carefully studied and in our last paper the kinetics of these phenomena at different temperatures are given.

BACQ: It is a great pity that mammals are not bacteria and could not be fitted into your data.

HOLLAENDER: Did you study any physiological or genetic phenomena?

POWERS: We were interested in morphological characteristics of the colonies and tried to get a deeper insight into the physical parameters which are of importance here. Very little attention has been given to other biological processes outside the scope of our investigation.

TARUSOV: Can nitric oxide be regarded as an inert substance with regard to the objects you studied?

POWERS: When oxygen is excluded the nitric oxide by itself is not toxic for bacteria. In other words the presence of nitric oxide produces no harmful effect on the spores; it does not affect them in any way. The same may be said for hydrogen sulphide.

I should like also to answer Dr. Gray's question. When we use nitric oxide, it must be carefully removed afterwards from the system. Otherwise some toxic phenomena may occur.

TOBIAS: From the survival curve it may be seen that a lethal effect is present. Can you determine the quantity of the radicals in one spore, in ten thousand or in other great quantity of spores? What is the precision of the experimental technique you have used?

POWERS: We are very happy that we can seek the advice of the physicists who use ESR methods.

PASSYNSKY: Is it possible to determine the radicals' concentration using more simple substances or some fractions of the spores studied? Or do they appear only when all the complexity of the spores' composition is retained?

POWERS: Of course, there is the possibility of carrying out these measurements on cellular fractions, but we have not done it. I may call your attention to the fact that quite recently in Dr. Gordy's laboratory there was observed, while studying caesin, an increase in the size of the doublet signals. It is almost identical to what I have shown today for spores.

PASSYNSKY: Was the quantity of the radicals determined directly under the beam?

POWERS: No. All the measurements were performed after the exposure and, by the way, in quite another building. Experimental material was transferred to other conditions.

BARENSEN: What could you tell about the cross-section and the effects of the linear energy transfer?

POWERS: Our experiments with irradiations of different ionization density were carried out together with Dr. Tobias' group in California using the HILAC. At present I can tell you that as linear energy transfer increases, radiosensitivity

changes, and the general outline of the survival curve depends on the ionization density. I would like to show our preliminary data but they are as yet too few.

ALEXANDER: Why do you think that ESR spectra are in some way related to chemical transformations that cause damage? When ESR measurements are taken, it is impossible to distinguish, whether the reactions in progress are important to the cell or whether they are trivial biological processes.

POWERS: It is very difficult to distinguish which mechanisms are more important and have crucial significance and which play a trivial role. I tried to present my material here very cautiously. I said that the role of the free radicals may be estimated from the biological effects. All the experiments listed above throw some light on the biological effects also. ESR data are only used as supporting evidence.

ALEXANDER: It is with satisfaction that I hear that ESR methods could help in the elucidation of biological problems. The data you have presented on the free radicals are in favour of this method and justify the hopes it arouses.

POWERS: My attitude towards ESR methods is very reserved since here we enter the sphere of physics. This method should be used very carefully and employed as a control. I do not consider this method a universal one.

# FLUORESCENCE STUDIES OF THE CHANGES UNDERGONE BY NUCLEOPROTEINS AND THEIR DERIVATIVES IN IRRADIATED CELLS

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## SUMMARY

Radiation-induced changes in nucleoproteins, nucleic acids and nucleotides in the cytoplasm and nuclei of living cells are described in the paper. These changes are detected on preparations vitally fluorochromed with acridine dyes, by fluorescent microscopy in the visible spectral region, as well as by an investigation of the auto-fluorescence registered in the ultraviolet spectral region.

The processes described represent labilization and denaturation of DNA-proteins, accumulation of RNA and nucleotides in the cytoplasm, and changes in their physico-chemical properties which have an effect upon the character of the fluorescence. The intensity and the spectrum of ultraviolet fluorescence of the cells in radiosensitive organs undergo considerable changes soon after X-irradiation.

Ultraviolet fluorescence microscopy applied to the study of radiation damage to cells yields, especially in combination with ultraviolet absorption microscopy and fluorescence microscopy in the visible spectral region, new facts about the state of cellular nucleoproteins, and their early and later changes due to radiation.

The first reports of the use of fluorescence microscopy in radiobiological studies appeared more than twenty years ago (Wels, 1938; Herčík, 1939; Biebl, 1942). Considerable advances made in this subject in the following twenty years extended its scope and led to a more knowledgeable use of the technique in various branches of biology, including radiobiology. Progress was facilitated firstly, by the introduction of fluorescent stains, fluorochromes, of low toxicity, suitable for vital and supravital investigations; secondly, by the development of fluorescence cytochemical methods, especially the cytochemistry of nucleic acids (Meissel and Korchagin, 1952; Armstrong, 1956; Schümmelfeder *et al.*, 1957; Bertalanffy and Bickis, 1956); and thirdly, by considerable improvements in the apparatus permitting fluorescence microscopy to be combined with phase contrast and ultraviolet absorption techniques (Brumberg, 1955; Haselmann and Wittekind, 1957). In this respect special mention should be made of the procedure



whereby the microscopic object is irradiated with incident light descending through the objective, a special fluorescence opaque illuminator being employed (Brumberg, 1955). Besides a number of advantages of a fluorescence nature in this case, considerable possibilities are laid open by the ability to observe one and the same site of an object simultaneously by phase contrast, dark field and ultraviolet methods and the determination of the character of the absorption.

## FLUORESCENCE MICROSCOPY IN THE VISIBLE SPECTRAL REGION

Strugger's (1940) observations on the specific behaviour of acridine orange towards healthy, pathologic and dead cells led Krebs (1947), Krebs and Gierlach (1951) and others to use this fluorochrome in radiobiological investigations. It was found in a number of cases, particularly with botanical material, that radiation-damaged cells accumulated larger amounts of acridine orange than normal cells and as a result emitted light of longer wave-length (yellow, orange or red). In 1953 Strugger, Krebs and Gierlach carried out studies on the early manifestations of injury caused by X-rays in plant cell cytoplasm.

In 1947 we began our studies which were centred on unicellular plants, cultures of animal cells *in vitro* and the hemopoietic organs of various animals. In this report we shall attempt to give a generalized survey of the results.

The first communications (Meissel and Zavarzina, 1947; Meissel *et al.*, 1951) pointed out that acridine orange binds differently with the nucleic acids of the nucleus and of the cytoplasm, imparting to these compounds fluorescence of different colour. In 1952 Meissel and Korchagin showed, on nucleoproteins isolated from the microbial cell, that deoxyribose compounds form green fluorescent complexes with acridine orange, whereas under the same conditions this same fluorochrome forms red fluorescent complexes with compounds of the ribose type. These findings, confirmed later by a number of other investigators, made it possible to determine with certainty the distribution and behaviour of nucleoproteins in irradiated cells under vital and supravital conditions.

The very first reactions towards radiation of cellular nuclei showing a light green fluorescence in the normal condition are manifested in a marked increase in the intensity of fluorescence of the nuclear membranes.

The delicate internal structures of the nuclei harden, the nucleolus and nucleoprotein granules swell, assuming a droplike or poorly appearance with increased intensity of fluorescence. It is at that time that a

characteristic separation of the nucleoproteins into two fractions begins to make its appearance. One fraction weakly fluorescent, has a green colour and the other fluoresces with a more brilliant, whitish light. At first the latter fraction is in close contact with the former, but gradually, evidently turning more fluid, it becomes mobile, circumventing the larger nuclear structures, nucleoli, nucleoprotein granules and the internal surface of the nuclear membrane.

The next stage is a sharper differentiation of the nuclear material. The amount of the brilliant white fraction increases, forming droplike accumulations that assume a greenish yellow colour and are often to be found touching the membrane. At this stage one may observe nodal enlargements of various shapes. At times this is accompanied by partial secretion of the brilliant white nucleoprotein fraction from the nucleus. The nuclear membrane becomes markedly thicker and begins to fluoresce with a yellowish green colour. The nuclear structures become undifferentiable. In separate areas of the nucleus may be found accumulations of the more fluid brilliant fraction in the form of two or three pools or droplets. In the next stage the nuclei disintegrate and in their place remain variously shaped accumulations of highly fluorescent brilliant white substance.

We believe that the changes observed in the nuclear matter are associated with the various stages of denaturation of DNA-protein, with its labilization and with the separation of DNA from the protein. The initial stages of DNA-protein denaturation are evidently accompanied on increase in complex formation with acridine orange with a resultant intensification of the fluorescence of the complex. The separation of DNA from the protein is characterized morphologically by the appearance in the nuclear chromatinic structures of a substance exhibiting a bright whitish-green fluorescence. Further intensification of the fluorescence and a shift of the colour in the longer wavelength direction is accompanied by liquefaction of the substance (clearly seen in the microscope), and by a fall in viscosity, as a result of which the substance flows around the nuclear structures, forming drops and pools. In this way vital fluorochroming of the irradiated cells allows one to observe directly the various stages of denaturation and depolymerization of DNA-protein.

Such in general are the impairments in the nucleoprotein structures of the nucleus proceeding at various rates depending upon the dosage and upon the time elapsing after the radiation, as well as upon the radiosensitivity of the cells. A highly interesting effect, brought to light only with the aid of fluorescence microscopy is the change in the nature of the fluorescence of the damaged cell nucleus, a considerable increase in

fluorescence intensity and a characteristic shift in the fluorescence spectrum. The change in colour of the fluorescence of the acridine orange complex may be brought about either by the so-called concentration effect, described as long ago as 1940 by Strugger or through formation of dimeric and trimeric cations of the fluorochrome, a process investigated by Zanker (1952). Both these effects in the cases under consideration depend upon changes in the physico-chemical state of DNA-protein or of DNA. Their binding with diaminoacridine increases even in the initial stages of denaturation. It increases particularly on depolymerization of the highly polymeric DNA, complexes being formed of the type of those given by RNA with diaminoacridine. According to the concepts being developed by Bradley and Felsenfeld (1959) the degree of aggregation of acridine orange cations when the dye is being bound with polyanions depends upon the configuration of the polymer.

It is highly probable that the passage of DNA from the rigid bihelical to the more mobile helical structure during denaturation is accompanied by intensification of the dimerization of acridine orange cations. This may serve as the explanation of the shift towards the right of the fluorescence spectrum exhibited by the complex formed. Whichever of the interpretations proves to be correct on further study one may even now assert that the changes in intensity and colour of the fluorescence of nuclear structures in irradiated cells are manifestations of important changes in the physico-chemical state of the nuclear nucleoproteins. These changes are evidently very widespread and may be observed both in the cells of yeasts and in a variety of animal cells. We have found them in the bone-marrow of totally and locally irradiated animals (Meissel and Sondak, 1955, 1956; Kondratjeva, 1956). They appear immediately after irradiation, with doses from 100 r upwards, in the form of individual cells or of cellular aggregates with sharply changed and brilliantly fluorescent nuclei. Such aggregates formed as the result of coalescence of the cells we have termed "micronecrotic." The number of such micronecrotic foci increases with the dose and the post-irradiation time (Figs. 1 and 2). The maximum number is found 6 hr after irradiation, following which they gradually disintegrate and are resolved. The substance binding the cells into micronecrotic foci energetically absorbs u.v. light with a wave-length of 280 to 254  $m\mu$  and is apparently nucleic acid (Bukhman and Kondratjeva, 1959). In bone-marrow cells of irradiated animals (900 r, 4 hr after irradiation) there is a marked increase in absorption of u.v. rays of wave-length 365  $m\mu$  (Kondratjeva and Bukhman, 1960).

Similar cellular changes of focal character are found in the spleen (Sondak, 1957) and in the lymph nodes and thymus of irradiated

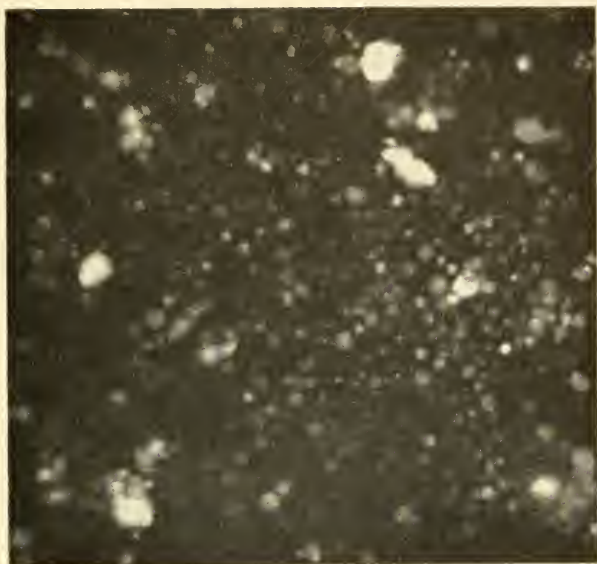


Fig. 1.—Micronecrotic foci in the bone-marrow of an irradiated rat (30 min after irradiation, dose 500 r).

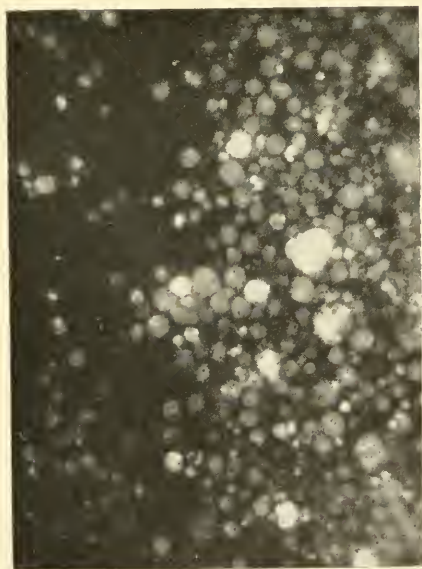


Fig. 2.—Micronecrotic foci in the bone-marrow of an irradiated rat (3 hr after irradiation, dose 500 r).



animals (Meissel, 1957; Meissel *et al.*, 1958). They result largely from the direct action of the radiation, since screening of the hemopoietic organs obviates the reaction to a considerable degree. The aforementioned nuclear impairments revealed by fluorescence microscopy were discovered soon after irradiation in leucocytes and lymphocytes of the peripheral blood vessels (Kondratjeva) and also after irradiation of blood *in vitro* (Kondratjeva and Pinto, 1961).

In the cytoplasm and small vacuoles of the cell acridine orange combines with the nucleic acid and nucleotides to form complexes showing a bright red fluorescence. This fluorochrome binds not only with existing (pre-formed) granules but also forms new ones as a result of the separating out of the dye-nucleic acid complexes.

Considerable amounts of nucleic acid compounds are observed to accumulate in the cytoplasm of irradiated cells. These compounds form with acridine orange numerous large and small granules showing a fiery red fluorescence. The large granules, or at least some of them, are distinguished by greater density and stability than the granules of unirradiated cells. They do not spread out under the action of various factors, including u.v. irradiation. The change in the nature of the cytoplasmic granules in the irradiated cells is indicative either of physico-chemical changes in the nucleic acids forming complexes with acridine orange or else of the formation of a stronger bond between these substances and the proteins of the cytoplasm.

Irradiated cells continuing their metabolic activities accumulate increasing amounts of nucleotides and ribonucleic acids in the cytoplasm, in a number of cases completely filling up the cells. This pertains equally to cells *in vitro* and *in vivo*, for instance the myeloid cells of the bone-marrow. It is interesting that in the cytoplasm and vacuoles of irradiated metabolizing yeast cells also basophilic substances and volutin accumulate, representing a complex of ribonucleic acid and polyphosphates. Hence retardation of the metabolism of nucleotides and other high energy compounds is a quite widespread reaction of irradiated cells, belonging to the most varied cellular types.

#### ULTRAVIOLET FLUORESCENCE

Many biologically important substances (aromatic amino acids, proteins, purine and pyridine bases, nucleotides, nucleic acids and some vitamins) possess absorption maxima in the u.v. region. In consequence one might expect that fluorescence of these substances could also be exhibited in the same spectral region. For a number of substances this has proved to be the case.



It was very interesting to ascertain whether the character of the u.v. fluorescence of organs, tissues and cells changes under the influence of ionizing radiation. This was studied at organic and cellular levels. In the present section we shall dwell only on the former. Khan-Magometova *et al.*, (1960) recorded the integral u.v. fluorescence spectra obtained from considerable areas of organs without taking into account the complexities of their tissue and cellular patterns. Thin sections of the organs of an animal (rat) or an homogenate were prepared on a freezing microtome. Blood plasma was also investigated. The homogenate of the organs, and the blood plasma, were placed in a special quartz cell 0.8 mm high, owing to which the objects under observation were all of constant thickness. Measurements of fluorescence were made with the aid of a photoelectric microspectrofluorometer designed by Agroskin and Korolev. The light source was a mercury arc lamp (DRSh-100) from the spectrum of which separate sections were isolated by means of a mirror monochromator with a diffraction grating. Rays coming from the monochromator were further passed through chlorine and bromine gas filters (when working in the region of 260 to 280 m $\mu$ ). The fluorescence spectra were analysed by a similar monochromator at the entrance slit of which a tenfold enlarged image of the object under investigation was produced with a quartz-fluorite micro-objective. The light intensity was determined by the magnitude of the signal from a photomultiplier mounted at the exit slit of the monochromator. Measurements were recorded as a deflection of a mirror galvanometer. The data were analysed in correspondence with the spectral sensitivity of the system micro-objective-monochromator-receiver.

The ultraviolet fluorescence peak of the organs investigated lies in the region of 320 to 330 m $\mu$ . Short wave u.v. irradiation causes a definite decrease in the fluorescence intensity and a gradual shift of the peak in the direction of the longer wave-lengths. With decrease in intensity of the initial peak there is an increase in intensity of a new peak of longer wave-length. This effect is undoubtedly due to active photochemical processes.

The fluorescence spectra of the organs of animals given whole-body irradiation with X-rays at doses of 1,000 r did not differ significantly from those of controls. However the intensity increased markedly in the radiosensitive organs (bone-marrow, spleen, lymph nodes) and in the blood plasma (as tested 4 and 24 hr after irradiation (Fig. 3)).

At present it is difficult to explain the increase in intensity of u.v. fluorescence of the radiosensitive organs. Perhaps this is associated with changes in the nucleoproteins or other proteins in which cyclic amino acids are liberated.

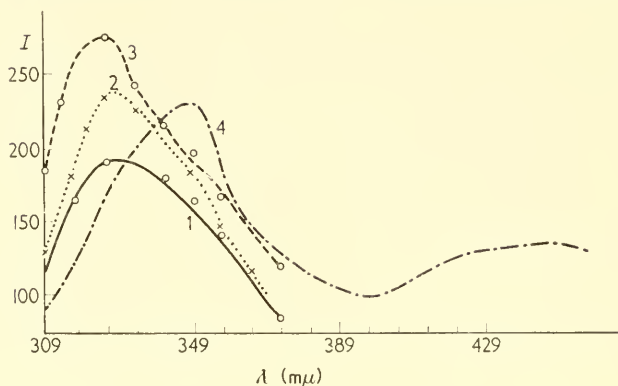


Fig. 3.—Ultraviolet fluorescence spectra of the blood plasma of normal and irradiated rats. 1—normal (control), 2–4 hr after irradiation; 3–27 hr after irradiation; 4—shift of spectra after u.v. irradiation.

With the aid of the photoelectric microspectrofluorometer described above Agroskin *et al.* (1960) investigated the low temperature fluorescence spectra of solid specimens of ribo- and deoxyribonucleic acids

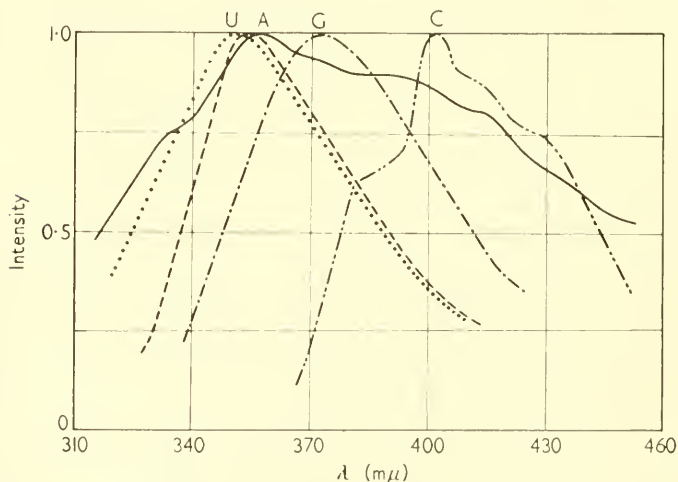


Fig. 4.—Fluorescence spectra of ribonucleic acid (from yeast) and of its component bases (excitation with  $\lambda = 260$  to  $280$  m $\mu$ ).

A—adenine, G—guanine, U—uracil, C—cytosine.

and their separate components. Of the data obtained the most interesting from the standpoint of the subject under discussion are the spectra of the nucleic acids and their bases (Figs. 4 and 5). It was found that the component bases of the nucleic acids and also the nucleosides and

nucleotides possess characteristic fluorescence spectra. The fluorescence of the nucleic acids covers the spectral region of emission of their bases and the difference in fluorescence of RNA and DNA is determined predominantly by the spectra of thymine and uracil.

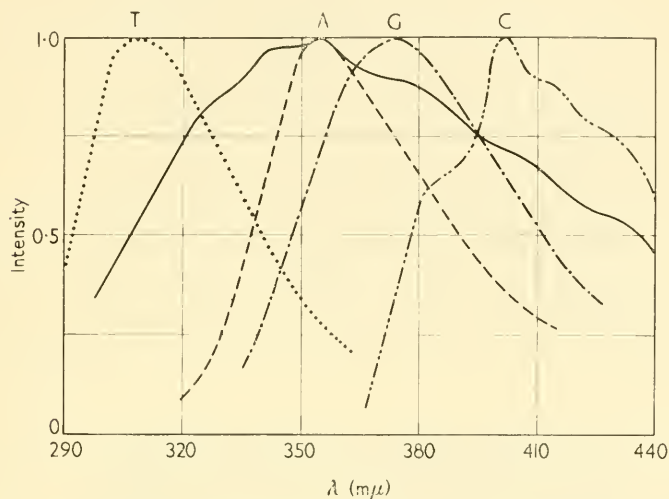


Fig. 5.—Fluorescence spectra of deoxyribonucleic acid (from the erythrocytes of hens) and of its component bases (excitation with  $\lambda = 260$  to  $280$   $m\mu$ ).

A—adenine, G—guanine, C—cytosine, T—thymine.

### ULTRAVIOLET FLUORESCENCE MICROSCOPY

In 1956 Brumberg described a new method of fluorescence microscopy which allowed one to photograph the u.v. fluorescence of microscopic objects. He made the assumption that the monotonous blue or violet autofluorescence of the majority of biological objects are the "tails" in the visible spectrum of a fluorescence of which the peaks are located in the u.v. region. This assumption has been confirmed. It was found that many tissues of plant and animal organisms possess characteristic and definitely expressed u.v. fluorescence (Brumberg *et al.*, 1958; Barsky *et al.*, 1959). The version of fluorescence microscopy in which excitation and recording of fluorescence is carried out in the u.v. region is called u.v. fluorescence microscopy.

Recently Brumberg and Barsky (1960) have described the application of the method to investigations of cytological objects. In this study, as well as in further investigations, the same site of the preparation was consecutively photographed in ultraviolet light, first in the light of its own u.v. fluorescence (on excitation by incident light coming through

the microscope objective) and then in transmitted light in order to ascertain the character of the u.v. absorption by the separate components of the object. The opaque illuminator over the objective had two interchangeable beam splitters of which one was characterized by high reflection of ultraviolet rays of wave-lengths  $280\text{ m}\mu$  or less and by good transmission of longer wave-lengths. This splitter was used for exciting and photographing the u.v. fluorescence of the specimen. The second beam splitter reflected u.v. rays in the range  $360$  to  $300\text{ m}\mu$ , transmitting light of the visible spectrum. It was employed in studies of visible fluorescence.

Excitation and recording of the fluorescence of the object was carried out with crossed filters. For u.v. fluorescence such filters were the following: a quartz cell with chlorine and bromine gas filters mounted between the light source and the microscope (transmits u.v.  $\lambda = 250$  to  $260\text{ m}\mu$ ) and a Woods glass filter (transmits u.v.  $\lambda = 340$  to  $380\text{ m}\mu$ ) mounted over the ocular of the microscope. Visible fluorescence of fluorochromed objects was observed respectively with blue and yellow filters.

The microscope employed in the work was fitted with a quartz-fluorite objective lens ( $58\times$ ) with an aperture of  $0.8$  (water immersion) and with a  $3\times$  quartz-fluorite ocular. The exposure during photomicrography of the u.v. fluorescence was 10 seconds and of absorption 2 to 3 seconds.

In a number of cases the specimens were preliminarily fluorochromed with very dilute solutions of acridine orange ( $I : 10^5$ ). Such treatment did not interfere with the photography of ultraviolet fluorescence inasmuch as acridine orange fluoresces only in the visible region. At the same time fluorochromed objects, particularly blood and bone-marrow cells and micro-organisms are more easily identified and discriminated and also focused better during subsequent photography in u.v. light.

A brief general account will now be given of the results of u.v. fluorescence studies of normal and irradiated cells arising from the joint work of Brumberg, Barsky, Kondratjeva, and their collaborators (1960) and of these authors in collaboration with Meissel and Gutkina.

Ultraviolet autofluorescence has been revealed under normal conditions in cells of the most varied types and origins, beginning with micro-organisms (bacteria, yeasts) and including the cells of higher animals and man. The fluorescence is particularly noticeable in cells cultivated *in vitro* (cells of human amnion, of monkey kidney, of HeLa tumour), in the cells of Ehrlich's ascites tumour and of a number of other tumours.

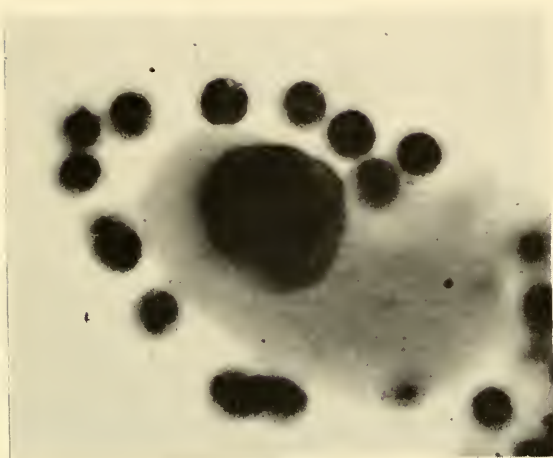


Fig. 6(a).



Fig. 6(b).

Fig. 6.—Ultraviolet absorption and autofluorescence of megakaryocytes and of cells of the myeloid series from the bone-marrow of a normal rat.

(a) u.v. absorption  $\lambda = 265 \text{ m}\mu$ .

(b) u.v. fluorescence.



In normal bone-marrow and blood distinct u.v. fluorescence is exhibited by cells of the myelocytic series up to mature leucocytes, inclusively; monocytes, megakaryocytes and thrombocytes. Young cells of the erythrocytic series and mature lymphocytes have weak fluorescence. Young lymphocytes (lymphoblasts) in the lymph nodes show clear u.v. fluorescence.

It is very significant that the fluorescent substance is found chiefly in the cytoplasm of the cells. It is either more or less uniformly distributed, or is concentrated for the greater part in definite regions of the cytoplasm, mostly those adjacent to the nucleus. In vital fluorochroming with acridine orange the substance fluorescing in the ultraviolet partially enters into the composition of the newly-formed granules.

Cell nuclei, evidently with some exceptions, do not exhibit marked u.v. fluorescence. At times nucleoli may fluoresce and a very faint diffuse fluorescence of the nucleus may be observed. But in these cases also the light emitted by the nucleus is incomparably weaker than that of the cytoplasm; as a rule the nucleus stands out as a dark object on a bright cytoplasmic background. This is somewhat unexpected. We have already indicated that deoxyribonucleic acid isolated from the cells shows a definite u.v. fluorescence. Evidently when it forms a component part of the DNA-protein complex deoxyribonucleic acid loses its capacity for fluorescence.

What is the nature of the substances responsible for the ultraviolet fluorescence of the cytoplasm? By means of special experiments on the selective extraction of various nucleic acid components from the myeloid cells, Brumberg *et al.*, (1960b) showed that they are nucleotides, ribonucleic acid and, to a lesser extent, proteins.

What then are the changes undergone by the u.v. fluorescence of cells subjected to u.v. and ionizing radiation? It was found that as a result of intensive irradiation by u.v. rays ( $\lambda$  250 to 280 m $\mu$ ) or X-rays (in doses exceeding 25kr) the u.v. autofluorescence considerably weakens giving place to clearly visible blue-violet fluorescence, excited by rays of 365 m $\mu$  wave-length. This fluorescence may be studied in an ordinary fluorescence microscope. It appears not only in the cytoplasm but also in the nuclei of the cells.

The sharp fall in u.v. fluorescence in intensively irradiated cells is due to the resultant photo and X-radiochemical processes leading to labilization of the nucleoproteins and release from the cells of nucleotides and nucleic acids. The appearance of a bright visible fluorescence is evidently associated with some kind of structural changes in the nucleoproteins and proteins, which have not yet been investigated more closely.

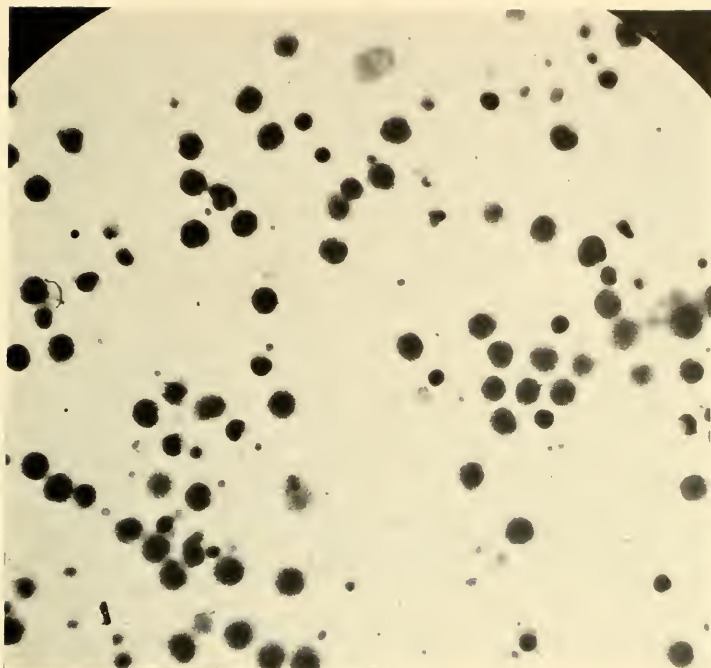


Fig. 7(a).

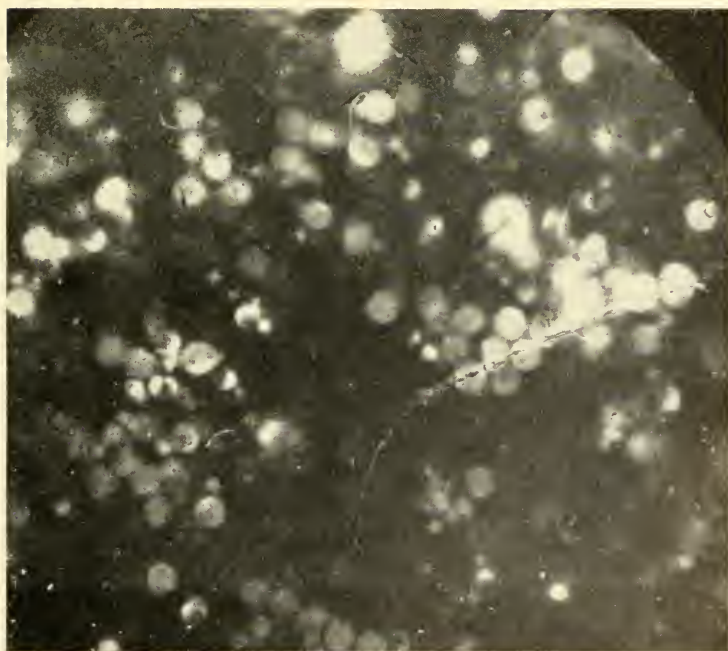


Fig. 7(b).

Fig. 7.—Ultraviolet absorption and autofluorescence of cells of the lymph nodes of a rat irradiated with a dose of 900 r, 4 hr after irradiation.

(a) u.v. absorption ( $\lambda = 265 \text{ m}\mu$ ), (b) u.v. fluorescence.

We now pass to a discussion of the studies on the u.v. fluorescence of the cells of radiosensitive and radiostable animal organs. In our experiments, white rats were irradiated with X-rays (by the apparatus RUM-3, 190 kV, 10 mA with 0.5 mm copper and 1 mm aluminium filters) in doses of 900 to 1,000 r, the dose-rate being 74 r/min. Cells of the various organs were investigated immediately, and 4 hr after, irradiation.

Already, soon after irradiation, a marked increase was observed in the intensity of ultraviolet fluorescence of the lymphocytes in the lymph nodes (Fig. 7) and in the blood and the fluorescence of the leucocytes. After irradiation there is a considerable increase in the u.v. fluorescence of cells of the myeloid series in the bone-marrow. Barsky obtained the fluorescence spectra of normal myelocytes and of the same cells from the bone-marrow of irradiated animals (Fig. 8). Examination of the spectra revealed considerable changes in those of the

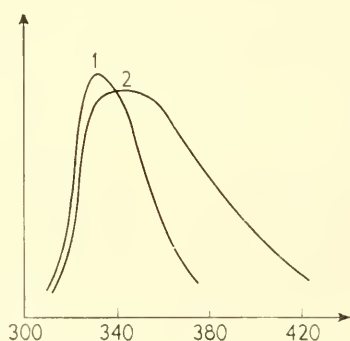


Fig. 8.—Ultraviolet fluorescence spectra of individual myelocytes: 1, from the bone-marrow of normal and 2, irradiated animals (rats) 4 hr after irradiation with a dose of 900 r.

irradiated animals. The emission bands broaden and are somewhat shifted in the direction of the longer wave-lengths. This bears evidence of qualitative changes in the substances responsible for the fluorescence. No changes in the character and intensity of the fluorescence could be detected in the liver cells either immediately after, a few hours after or 24 hr after irradiation. Even on the 9th or 10th day after irradiation with a dose of 500 r the liver cells fluoresced in the same way as the controls.

The necrobiotic changes of the cells in the bone-marrow and lymph nodes of irradiated animals, which we had described previously as micronecrotic and which are clearly observed on fluorochroming with acridine orange (on fluorescence microscopy in the visible region), were

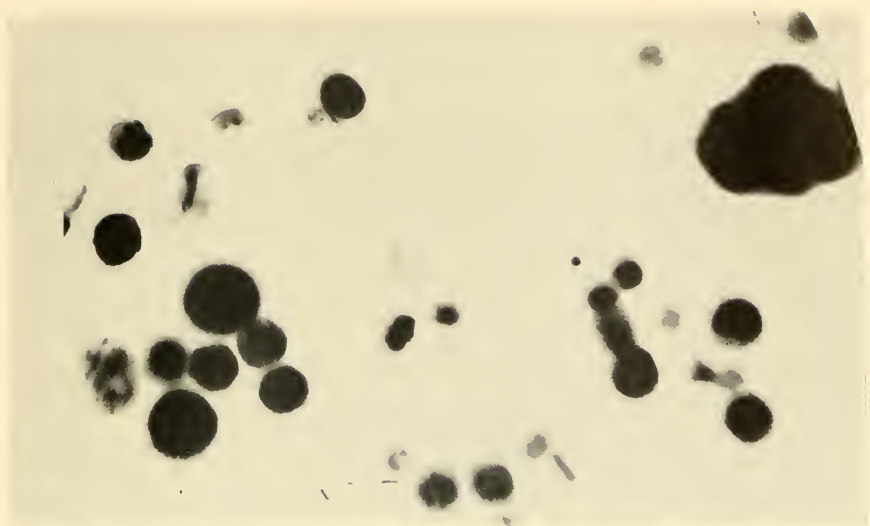


Fig. 9(a).

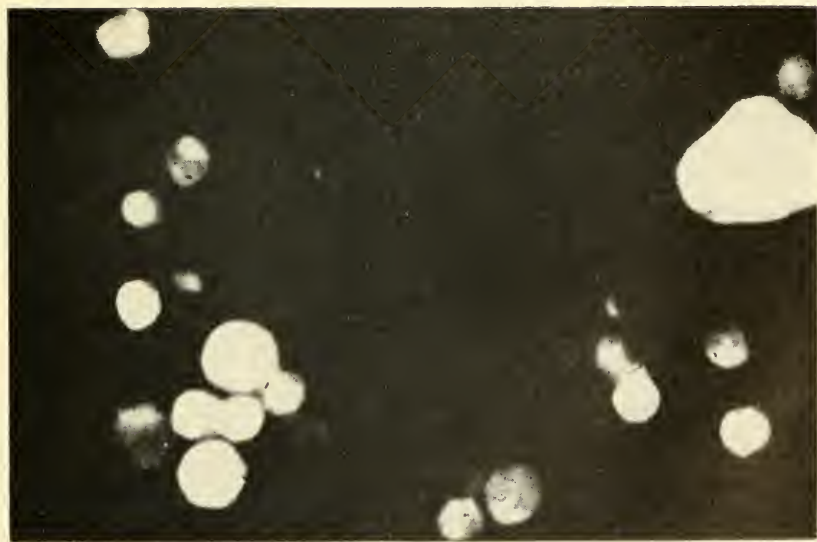


Fig. 9(b).

Fig. 9—Ultraviolet absorption (a) and autofluorescence (b) of bone-marrow cells of an irradiated rat (dose 900 r, 4 hr after irradiation). In the upper right hand corner is a micronecrotic focus.

distinguished by a highly brilliant u.v. autofluorescence (Fig. 9). The substance responsible for this fluorescence is nucleoprotein modified in a specific manner and firmly attached to the cellular substrate from which it is separated with difficulty.

We also observed enhanced fluorescence in irradiated animal cells continuing their growth and metabolism on cultivation *in vitro*. This was observed already after a dose of 100 r and attained a maximum at doses of 2,000 to 3,000 r (Fig. 10). Further increase in dosage led to a diminishing of the u.v. fluorescence and at 25 to 30 kr it became extinct giving place to higher wave-length emission, in the visible region.

The increase in the ultraviolet fluorescence of irradiated (with moderate doses) cells continuing to metabolize parallels the accumulation in them of nucleotides and ribonucleic acid and these processes are presumably intimately connected. The newly-formed RNA obviously differs from the normal one. In certain types of cells (for instance HeLa cells) after irradiation marked u.v. fluorescence appears in the nucleoli.

It should be stressed that cells irradiated with X-rays and continuing their metabolic activities are highly susceptible to irradiation by short u.v. waves. Such irradiation very quickly leads to extinction of u.v. fluorescence and to the appearance of longer wave visible fluorescence. We observed this in cultures of monkey kidney cells *in vitro* irradiated with doses beginning from 100 r.

In irradiated yeast cells (*Endomyces magnusii*) continuing to metabolize on a nutrient medium we observed a well-defined u.v. autofluorescence of the nuclei. In non-irradiated cells the nuclei as a rule do not exhibit fluorescence (Fig. 11). This is the only case we have observed of the appearance of u.v. fluorescence in the nuclei of irradiated cells.

## CONCLUSIONS

1. Vital and supravital fluorochroming followed by observation in the fluorescence microscope permits detection of the early stages of impairment in the nucleoproteins of cell nuclei beginning immediately or soon after irradiation. These are revealed as a result of the changes in the nature of the complex formed between diaminoacridine fluorochromes and structurally damaged DNA proteins, affecting the intensity and colour of the fluorescence of the newly-formed complexes. The nature of such structural impairments is not yet completely elucidated. Possibly labilization and breakdown of the DNA linkage with



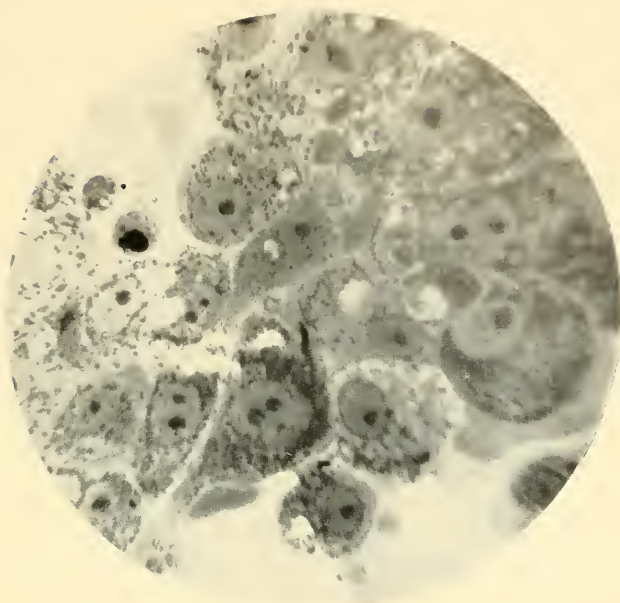


Fig. 10(a).

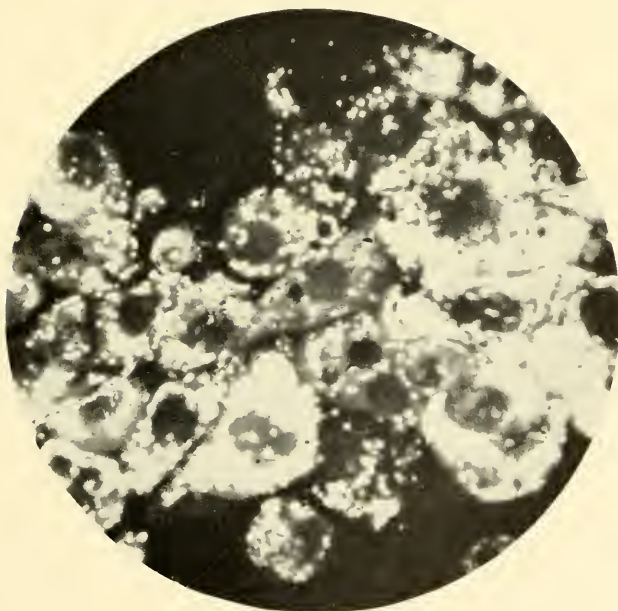


Fig. 10(b).

Fig. 10.—Ultraviolet autofluorescence (b) and absorption (a) in the kidney cells of a monkey (culture in vitro) 48 hr after irradiation (dose 2,000 r) ( $\lambda = 265 \text{ m}\mu$ ).



Fig. 11(a).



Fig. 11(b).

Fig. 11.—Yeast cells (*Endomyces magnusii*) after irradiation and subsequent cultivation.  
(a) u.v. absorption ( $\lambda = 265 \text{ m}\mu$ ). (b) u.v. fluorescence.

the protein (partial deproteinization) and the initial stage of DNA denaturation take place with transition of the DNA to a less rigid structure. This in turn facilitates the aggregation of the cations of acridine orange bound with DNA (*cf.* Bradley and Felsenfeld, 1959).

Fluorescence microscopy in the visible region of the spectrum reveals initial and progressive changes in the physico-chemical state of DNA, in particular its separation from the chromatinic structures in the irradiated cell nuclei and depolymerization. The changes may be detected soon after irradiation in the hemopoietic organs and in the leucocytes of the peripheral blood stream.

2. By means of vital and supravital fluorochroming in hemopoietic organs of irradiated animals one may observe peculiar focal cellular degenerations—"micronecrotic foci". The nucleic acids of cells forming such foci acquire enhanced affinity for diaminoacridines and brilliantly fluoresce both in the visible (on fluorochroming) as well as in the ultraviolet (primary fluorescence) regions of the spectrum. The physico-chemical properties of the nucleic acids and the nature of their bonds with the cellular structures evidently differ significantly from normal.

3. In the cytoplasm of irradiated cells considerable accumulation of nucleotides and nucleic acids takes place, some of them evidently differing from the normal. These substances possess a clearly defined ultraviolet autofluorescence, as a result of which in irradiated cells that continue their metabolic activities there is a marked intensification of ultraviolet fluorescence. The fluorochrome acridine orange forms complexes with nucleotides and RNA that separate out in the form of granules with a red fluorescence. The content of such granules increases to a great degree in irradiated cells. They fluoresce (autofluorescence) also in the ultraviolet region. Some of the granules in irradiated cells are characterized by increased stability. On the other hand the majority of the cytoplasmic nucleoproteins of irradiated cells become very labile and sensitive to the short wave ultraviolet spectrum.

4. Ultraviolet autofluorescence of animal cells and organs and of blood plasma quickly diminishes under the influence of intensive ultraviolet ( $\lambda = 250$  to  $280 \text{ m}\mu$ ) and X-ray irradiation owing to the occurrence of photochemical and X-ray processes. In parallel this fluorescence appears in the longer wave-length, visible part of the spectrum. The appearance of this fluorescence is a sign of major changes in the structure and state of the nucleoproteins and proteins.

5. Ultraviolet fluorescence microscopy (Brumberg, 1956) first applied to the study of radiation damage to cells yields particularly in combination with ultraviolet absorption microscopy and fluorescence

microscopy in the visible region, new facts on the state of cellular nucleoproteins, and on their early and later changes due to radiation.

It has been found that the brightness and spectrum of ultraviolet autofluorescence of the cells of radiosensitive elements change significantly after X-ray irradiation. The changes are most likely connected with alterations in the state of the cellular nucleoproteins.

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## DISCUSSION

ERRERA: I am interested in this fluorochroming of the cells. Were they living cells which were stained?



MEISSEL: In all the experiments on fluorochroming shown here vital or supravital staining was performed.

MARCOVICH: What was the concentration of the acridine orange? Did the staining of the medium occur? How many generations of the yeast cells did you observe at the concentration used?

MEISSEL: Acridine orange diluted 1:100,000 or 1:50,000 in physiological saline or in the medium for animal cell cultures was used. Acridine orange blocks cell structures which results in inhibition of the normal division of the yeast cells. When the concentration of the stain is not very high, the vitally fluorochromed yeast cell, being transferred to a fresh culture medium, would first of all free its structures from fluorochrome, excrete the stain and only afterwards begin its multiplication.

SHABADASH: According to your microphotographs radiation effects, as evidenced by autofluorescence, are found both in radioresistant (kidney) as well as in radiosensitive (haemopoietic organ) cells. Can we conclude that all the cell types in the body are radiosensitive? When absorption and fluorescence in the ultraviolet light of the kidney culture cells is studied mitochondria are discernible which also show autofluorescence. There is an increase of this effect following irradiation. How should we consider the part which ribonucleoproteins play in the overall cytoplasmic reaction?

MEISSEL: Tissue culture cells are somewhat more radiosensitive than similar cells in the body. Here we showed cells that had received doses of 2,000 r. Strictly speaking all cells are radiosensitive, but they differ as to the degree of their sensitivity.

I believe that among those cell nucleoproteins which begin to show bright fluorescence in the ultraviolet region following irradiation, there are mitochondrial ribonucleoproteins, but probably also some other types of the cytoplasmic RNA as well as nucleotides.

TOBIAS: Did you observe any changes in these same cells under the microscope some time after exposure? Did not the photodynamic effect of the acridine orange manifest itself in the cells? What changes occur there when you expose them to light for a long time while taking the photographs.

MEISSEL: In our work we tried to achieve vital fluorescence of the cell. For this purpose fluorescence of the cells fluorochromed with acridine orange was excited not by ultraviolet, but by blue-violet light, which is much less dangerous than ultraviolet. Apart from this, much depends on the exposure during observation and taking photographs. Exposure in our work was short, and no distinguishable changes caused by induced fluorescence were observed. When observation was carried out for a longer time distinct photodynamic reactions manifested themselves in the cells.

ZEITLIN: Did any changes occur in the absorption in the short wave ultraviolet region following formation of the complex acridine orange + nucleic acid?

MEISSEL: The fact is that acridine orange absorbs ultraviolet light in the same region as nucleic acids. As to whether there are changes in the absorption and fluorescence due to complex formation: nucleic acid—diaminoacridine—this is a problem we are studying at present. Important data on this problem have been



obtained by De Bruyn and co-workers (1953) Morthland *et al.*, (1954), Bradley and Felsenfeld (1959) and others.†

KUZIN: How soon after the irradiation could the shift of the fluorescence maximum be shown?

MEISSEL: Practically immediately following exposure.

GRAY: I would like to ask about the shift of the ultraviolet autofluorescence maximum, which occurred following irradiation with 25,000 r. In order to obtain this effect is it necessary to use so high a dose, or could you observe it at lower dose levels?

MEISSEL: Unfortunately we have not yet studied the dose dependance of the effect. But just after irradiation beginning with doses of 25,000 r this effect was observed, while at the doses of the order of several thousand roentgens we did not observe it.

PASSYNSKY: What is the relative role of the chemical and physical alteration of the nucleic acids or nucleoproteins in the ultraviolet fluorescence changes? Do similar changes occur in pure RNA and nucleoprotein preparations or do they become manifest only at the cellular level?

MEISSEL: First may I answer the second question? At present we have at our disposal only preliminary observations regarding ultraviolet fluorescence of the isolated nucleic acids, including RNA. Ribonucleic acid isolated from irradiated micro-organisms shows earlier changes in the pattern of its ultraviolet fluorescence, induced by short wave ultraviolet light, than does RNA from non-irradiated cells.

As to the first question. Here, probably, both types of change occur. It is difficult to say which are prevalent, but probably owing to configuration changes of DNA molecules during its denaturation, an additional capacity for binding acridine orange appears. Initial depolymerization processes are also evidently liberating some free bases which are capable of binding acridine orange; this is one possibility; another possibility lies in the fact that a changed DNA configuration or the transition from two-helix to one-helix form brings about more active formation of dimer and trimer complexes of the acridine orange.

PASSYNSKY: But these structural changes require some time, while in your experiments they were observed immediately.

MEISSEL: "Immediately" is but relative. Since it is difficult to make observations directly under the beam, there was a lapse of several minutes and sometimes 10, 20 or even more minutes between irradiation and the recording of the effect.

TUMERMAN: You have pointed out that most probably the physical cause for the changes in the fluorescence spectrum of the acridine orange consisted in differences in its concentration. A phenomenon is known of a fluorescence spectrum change in the long wave range occurring with the increase of the concentration, i.e. with the increased interaction between molecules of a substrate and the stain. Could the possibility be ruled out that configuration changes allow the development of triplet long term luminescence? In the preliminary experiment we

† DE BRUYN, P. P. H., FARR, R. C., BANKS, H., and MORTHLAND, F. W. (1953). *Exp. Cell Res.* **4**, 174. MORTHLAND, F. W., DE BRUYN, P. P. H., and SMITH, N. H. (1954). *Exp. Cell Res.* **7**, 201. BRADLEY, D. F., and FELSENFELD, G. (1959). *Nature, Lond.* **184**, 1920.

carried out together with you this possibility was evidently not confirmed. The spectrum of the orange luminescence of the acridine orange adsorbed to biological substrate is identical with that of a very concentrated water solution, while the spectrum of the green fluorescence coincides with that of the diluted water solution of this stain. There is at present also no indication of the change of the duration of the excited states.

Thus the physical mechanism of the observed changes consists probably in a greater or lesser proximity between the adsorbed stain molecules, i.e. a greater or lesser degree of interaction between molecules of the stain adsorbed by the biological substrates, and a greater or lesser amount of the stain.

GRAY: In relating the changes to the time, should it not be measured starting from the beginning not from the end of the exposure. What was the irradiation dose-rate in your experiments?

MEISSEL: In the case of the total body irradiation the dose-rate was about 74 to 75 r/min, i.e. all the dose was administered to the animal during about 15 minutes. When tissue cultures were irradiated it was possible to use irradiation of a very high dose-rate of the order of 20,000 to 25,000 r per minute.



# A DISCUSSION OF SOME IMMEDIATE EFFECTS OF X-IRRADIATION ON LIVING CELLS

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## SUMMARY

Attention is drawn to a few of the immediate biochemical effects of X-rays mentioned in the literature.

Some of these may be due to radical or direct attack on enzyme systems or cell structures.

One difficulty in discussing the initial effect of irradiation on cells is to decide what we mean by this. Our target is liable to be that event in which we are most interested at the time and, in any case, our ideas are usually conditioned and limited by the ease of detection of the effect; in fact, by its manifest importance to the cell.

Normally, also, we are considering effects which are irreversible or only slowly reversible. By using special techniques of killing cells during the course of irradiation, rapidly reversible changes can sometimes be seen, for instance in the proportions of oxidized to reduced co-enzyme. One is inclined to think that such changes and many reparable cytoplasmic injuries are unimportant. It seems possible that, though no immediate dramatic effect is usually to be seen, part of the phenomenon of ageing by irradiation may be the result of such causes.

An immediate dramatic effect can be shown in the result of the irradiation of the sporangiophore of *Phycomyces*. Forssberg (1960) found that the elongation of the sporangiophore could be inhibited temporarily by doses as low as 0.0005 r, a maximal effect being produced by 1.0 r. The inhibition is quickly reversible when irradiation is stopped. The mechanism of elongation is not fully known and it is difficult to picture in what way it could be affected by such tiny doses. If an enzyme or specific chemical substance is concerned, then it must surely be directly connected with the elongating cell wall or the water-imbibing membrane and some energy-transporting mechanism must be imagined. Forssberg has found an accumulation of lactic acid during this period of inhibition of elongation and also an abnormal distribution

of organic phosphates. This could be a very interesting system for studying an initial reaction and its final visible effect.

The manifest effect of irradiation on the cell nucleus and the consequences to cell life have made us regard this as the main target and think of initial effects as being within the nucleus; occasionally, however, events caused by simple enzyme inhibitions, probably unconnected with the nucleus, obtrude themselves upon our notice.

A striking example is given by the work of Gordon (1956) who found that the growth of young mung bean plants could be stunted for one or more days by low doses of X-rays. He traced this effect to an immediate fall in the auxin content of the irradiated plant. This in turn was found to be due to a considerable decrease in the usual formation of auxin from tryptophan, probably because of an inhibition of the enzyme responsible for the last stage of the transformation. Gordon considered that the enzyme responsible was that causing the oxidation of indole-acetaldehyde to auxin and could show that extracts made from bean plants *immediately* after irradiation showed a lower enzyme activity than extracts from normal plants. The initial action of the X-rays, biologically speaking, was apparently the inactivation of this enzyme, but it is not yet possible to say what, in its structure or surroundings, made it particularly sensitive.

Another interesting case of the obvious manifestation of the result of irradiating an enzyme system is given by Ungar *et al.* (1955) who studied the irradiation of an adrenal gland during its perfusion with blood containing A.C.T.H. At the end of the experiment, when the total dose had reached 2,000 r, it was found that the usual production of adrenal steroids had been much reduced. It was possible to show that definite steps in the formation of these steroids had been inhibited and the effect must have been on specific enzymes or on the formation of these enzymes.

To come at last to events in the nucleus, it has, of course, struck all workers that the huge nucleoprotein molecules offer a target that cannot be missed and that some of the initial effects of irradiation must be within them. This idea will be discussed later, but it is worth pointing out that it has proved easier to produce changes in other mechanisms connected with the mitotic process than to show any such effect.

For instance, it was deduced by Pele and Howard (1953) from autoradiographs and later demonstrated directly by ourselves on the regenerating rat liver (Holmes and Mee, 1955) that fairly small doses of X-rays given before the synthesis of deoxyribonucleic acid had started, were effective in stopping this synthesis. Important work by Bollum and Potter (1960), supported by the independent evidence of Ord and



Stocken (1960), showed that the real effect was an inhibition of the formation of the enzymes needed to produce the synthesis of the nucleic acid. If this effect on the formation of adaptive enzymes is likely to be a usual point of attack by X-rays, it must be further studied. It is unfortunately not yet known whether, when such enzymes appear in the tissue in response to a new need for them, they represent a new or much increased formation or actually an uncovering of enzymes already present. That this type of uncovering of enzymes or removal of enzyme inhibitors can occur in tissues has been demonstrated in various cases. Schneider (1960), however, has been able to cause an inhibition of nucleic acid synthesis in regenerating liver by giving inhibitors of protein synthesis at this early stage. It may be that, in his experiments also, the appearance of new enzyme is being prevented, and therefore, that this new appearance is indeed a new formation of protein.

Another system of synthesis, this time actually in the nucleus is the phosphorylation of nucleosides demonstrated by Osawa *et al.* (1951) in isolated thymus nuclei and shown in Dr. Stocken's department to be extremely easily inhibited by previous irradiation of the animal. It was difficult to demonstrate the existence of the phosphorylating enzyme in various other tissue nuclei but recent work by Crathorne and Shooter (1960) on the whole animal suggests that a phosphorylating enzyme exists in the nuclei of ascites tumour cells and of regenerating liver cells at some early stages of regeneration. When thymidine is injected into the animal, mono-, di- and triphosphate derivatives are found in the nucleus in much greater amounts than in the cytoplasm. It will be most interesting to know whether this system is also a primary target of X-ray action. Osawa *et al.* found that, as would be expected, the existence of a phosphorylating system is necessary for the formation of protein in the isolated nuclei.

We have lately been following the uptake of amino acid into one fraction of the nuclear protein and attempting to trace the effect of irradiation upon this and upon nucleic acid synthesis at the same time. For this we have done experiments *in vivo* with rat liver in the condition of regeneration after partial hepatectomy.

The animals were taken at a time after hepatectomy when nucleic acid synthesis was already occurring actively and when a large X-ray dose (2,000 to 3,000 r) was necessary to reduce it to 50 per cent of the normal rate. In our laboratory, Looney *et al.* (1960) demonstrated that this inhibition represented a slowing of synthesis only and that the irradiated cells eventually formed their full complement of DNA. To do this, they needed about 13 hr instead of the usual 8.

The figures are taken from Dr. Looney's paper. He gave 3000 r to the regenerating liver lobe (a dose which had been shown to reduce  $^{32}\text{P}$  uptake into the DNA to about half the normal amount) the rest of the body being shielded as well as possible. Tritiated thymidine was given immediately after irradiation and the animal killed 3 hr later. Figure 1 is a histogram showing grain count distribution. The irradiated cells

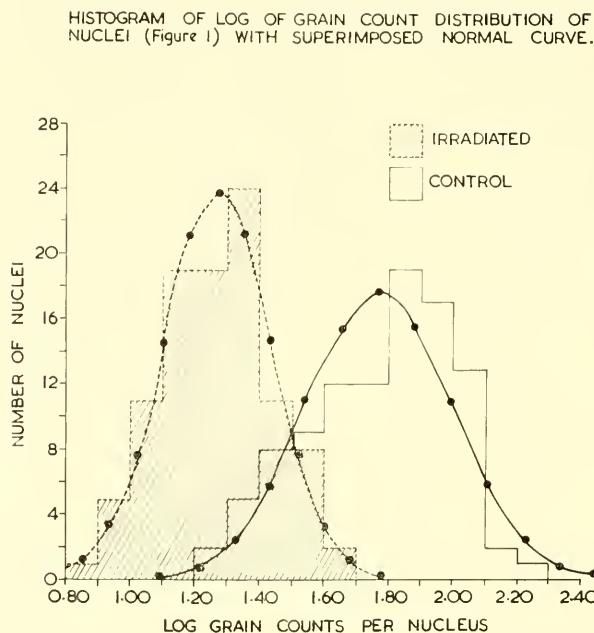


Fig. 1.—Histogram of the logarithm of grain count distribution of nuclei with the normal curve superimposed.

have a smaller grain count than those from an unirradiated but otherwise exactly comparable animal but there is no decrease in the number of cells in synthesis. At this time after hepatectomy no mitosis has yet occurred in either cell population. The reduction in grain counts in the irradiated cells might indicate a lower rate of DNA synthesis. In order to prove that this was the correct interpretation, measurements were made of the content of DNA in individual cells by a light absorption method using Feulgen-stained cells. Figure 2 shows the results. Unlabelled, that is to say non-synthesising, cells indicated the  $2n$ ,  $4n$  and  $8n$  amounts of DNA. Most hepatocytes in these animals contained a  $4n$  amount which increased to an  $8n$  amount before cell division. Cells which were synthesising DNA at 17 hr after hepatectomy become

labelled and are indicated here by black dots. The figure shows that the normal cells had all attained the full  $8n$  amount of DNA 6 hr after labelling whereas the irradiated cells had only completed about half their synthesis.

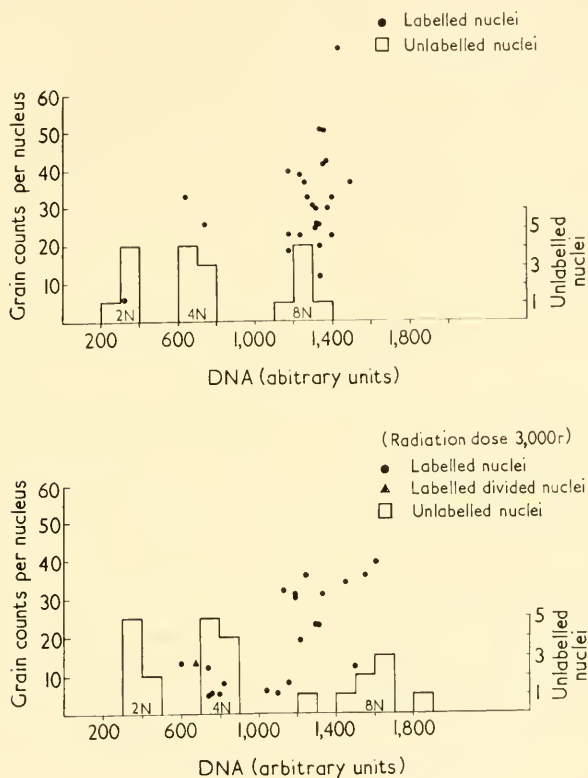


Fig. 2.—Plot of grain counts against DNA content of the same hepatic nuclei of a rat given tritiated thymidine at 17 hr after hepatectomy and sacrificed 6 hr later.

In absolutely similar material we have now made a simultaneous study of the synthesis of nucleic acid and of the residual protein to which the nucleic acid is found to be attached after removal of the histones. The synthesis both of this protein and the histones normally seems to take place at much the same time as the synthesis of the DNA in regenerating liver, since the uptake of lysine and of arginine into the proteins is then three or four times higher than that in resting liver tissue.

We have not nearly enough data to make any statement about the radiosensitivity of the formation of this particular protein. We can, however, say that in many individual animals, the X-rays have been sufficient to cause a sharp inhibition of nucleic acid synthesis without causing any inhibition of synthesis of this particular protein fraction, which in normal cells apparently occurs at the same time as the nucleic acid synthesis. The effect of X-rays appears therefore, to cause some dislocation in the usual relationship between the two.

The work of Richards (personal communication), who has made measurements on individual cells, is in agreement with the idea that the rate of protein formation is not depressed together with the rate of deoxynucleic acid formation.

After these diversions one must, however, return to a consideration of the effect of X-rays on various nuclear structures, since there is no doubt of the great importance of these effects in dividing cells or resting cells that may later come into division.

The nucleolus may, perhaps be considered as the prime target for the inhibition of synthesis in the nucleus at some stages of the mitotic cycle. Seed, (1960), in our laboratory, irradiated the three nucleoli in the nuclei of mouse heart fibroblasts with about 500 rad X-rays. The X-rays were collimated into a thin pencil  $1\mu$  in diameter. Two daughter cells were chosen from a previous division; one of them was used as a control and the other irradiated about 2 hr after the division. The progress of DNA synthesis in these nuclei was followed by taking an ultra-violet photograph after the chosen period of time had elapsed. It was found that synthesis had occurred in the control nucleus but not in the irradiated nucleus at times of 3 to 7 hr after the irradiation.

If we wish to speak of an initial effect we should obviously have grounds for considering it to occur immediately after irradiation; in a technique such as Dr. Seed's this cannot be demonstrated. However, Gaulden and Perry (1958), working at Oak Ridge, have been able to demonstrate immediate cessation of mitotic progress after nucleolar irradiation with u.v. light in very early prophase. Later stages of mitosis were not so sensitive to nuclear irradiation so that here we may consider our effective target to have changed.

The nature of the X-ray injury to the nucleoprotein complex of the cell has not proved easy to detect. The extraction of nucleoproteins from irradiated and unirradiated cells for comparison of their physico-chemical properties is not absolutely satisfactory because such small changes in technique cause variations in the extracted products. The control sample may show different viscosities, different abilities to form gels and so forth if the speed or duration of homogenization of the

tissue, for instance, is varied. Dr. Ruth Itzhaki has been carrying out work of this sort, and has found that the description of extraction methods given in the literature are often quite inadequate because such details are not given. For instance, when homogenizing mouse spleen at fairly high speed in normal saline, she found that insoluble and probably denatured nucleoprotein fibres were formed, while at slow speeds whole cells were still present and no fibres. Reproducibility was difficult because the resistance of the homogenate reduced the speed of the homogenizer by an unknown amount. These facts are mentioned merely to illustrate the difficulties of such work with nucleoprotein complexes.

Many experiments reported in the literature give no information about the initial lesion produced by the irradiation, since the nucleoprotein extraction is not made immediately after irradiation.

Perhaps the most definite evidence of damage to this structure by irradiation has been given by the work of Ord and Stocken (1960), who used the Bendich method of separating different fractions of the DNA and found that irradiation caused an increase in the amount of the more soluble fractions at the expense of the less soluble.

Irradiation *in vitro* of already extracted nucleoprotein must give different results according to the method of extraction used and one is then faced with the difficulty of deciding which preparation most nearly resembles the substance as it occurs in the living cell. To give a rather extreme example Anderson and Fisher (1960) found that the extraction of rat thymus brei with 2 volumes of 1.4 M sodium chloride produced a sample of very high viscosity. The authors considered that linear aggregates of DNA were present, other linkages having been broken by the strong salt. As expected by Anderson, these complexes were very sensitive to irradiation and a definite fall in viscosity was noted after a dose of 50 r.

The kindness of Professor J. S. Mitchell allows me to present to you some partly unpublished data of his own (Mitchell, 1959) which deal with a true initial effect and possibly one in which the deoxyribonucleic acid is involved. The Walker rat carcinoma has been irradiated *in vivo* in an anaesthetized animal with 2,000 r given in 2 min 16 sec. By a special device, liquid nitrogen is applied to the tumour less than 1 sec after irradiation. On warming the excised tissue high energy u.v. quanta may be released mainly at  $-5^{\circ}\text{C}$  and 50 to  $70^{\circ}\text{C}$ . In 35 recent experiments definite results have been observed in 5 cases and possible ones in a further 6 cases. The long-lived excited states must last for some seconds or even minutes at body temperature and might be associated with reactive chemical intermediates, but the experimental results are not inconsistent with an excitor mechanism. If the DNA is con-



cerned in this the energy could probably be utilized in some part of the DNA or be available for release in the protein closely associated with it. The secondary effects of such an initial process could be different according to the exact condition of the DNA. The irregular chromosome structures sometime found in tumours, virus-infected nuclei, chromosomes with puff formation, DNA during the process of reduplication, and so on, might suffer changes rather different from the usual. Professor Mitchell himself is trying to determine whether the findings can be related to the radio-curability of the tumour. At all events, there is here a chance of seeing an absolutely initial effect and of investigating its real meaning and relating it to the results which follow later.

#### ACKNOWLEDGEMENTS

I am most grateful to the Wellcome Foundation for providing my travelling expenses to this meeting.

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#### DISCUSSION

TUMERMAN: I am very interested in the information on Prof. Mitchell's discovery of ultraviolet emission during warming of frozen tissues. I would like to know whether the temperature dependence of the intensity of this luminescence was studied and what is Prof. Mitchell's or your opinion on the physical mechanisms of the origin of this phenomenon. Are you inclined to consider this luminescence as a chemi-luminescence attendant upon a free radical recombination process, or some oxidation reaction, or as a phenomenon of thermoluminescence resembling the known thermoluminescence of chloroplasts discovered by Arnold and Sherwood?

HOLMES: I know that the emission occurred at different temperatures, both at 5° and 50° C. Prof. Mitchell's hypothesis is that one of these processes is associated with the presence of long-lived intermediary chemical products, for instance hydrogen peroxide. The other one in his opinion may be due to DNA molecules; the energy released is passed either into the surrounding medium or to other DNA molecules. His opinion is not more definite than that. This is all that I can tell about these experiments.

MOUTON: Is radiation damage to the cell accompanied by an increase in the lactic acid content?

HOLMES: In Dr. Forssberg's experiments a temporary rise of the lactic acid content was observed at a time when increase in length of the sporangiophores was inhibited. Afterwards the lactic acid was removed.

ERRERA: I am interested in Prof. Mitchell's study. I would like to ask whether you know what the emission spectrum was?

HOLMES: No, I do not know that. Prof. Mitchell told me only that it was in the short wave ultraviolet with unexpectedly high energies.

POWERS: Which cells have you studied when nucleoli were irradiated by microbeams of X-rays?

HOLMES: Fibroblasts of the mouse heart. Every one of the three nucleoli in these cells was irradiated. This study was made by Dr. Seed.

BARENSEN: What was the irradiation applied to the nucleoli?

HOLMES: Approximately 500 r per nucleolus. I myself have never used this equipment and cannot give an exact description of it here.

MANILOV: You have told us that if, after the radiation exposure, mononucleotides are added, DNA synthesis continues but is delayed by 13 hr. Could you tell us which nucleotides should be added and how in your opinion these nucleotides penetrate into living cells and are taken up by the nucleus?

HOLMES: We have never attempted experiments with nucleotides. We do not know what is the cause for this delay of synthesis, but according to some investigators it is due to damage to the template or to some part of it in any case. It is possible that the damaged part is less protected than the nucleoprotein molecule as a whole. My personal opinion is that the delay may also be due to some interference with the phosphorylation process connected with nucleotides. Dr. Looney did not administer nucleotides but only thymidine.

#### Addendum to Discussion by Dr. Barbara E. Holmes

It is known that mitosis can be inhibited by mechanisms other than the inhibition of DNA synthesis, since a fairly small dose of X-rays will prevent cells from passing into mitosis even when the dose is given after DNA synthesis has been completed in those cells. We can illustrate this point with regenerating rat liver, since a dose of 450 r given to the dividing tissue immediately prevented any more cells from passing into mitosis—but I do not know how long this effect lasts.

At the other extreme 450 r given to cells before DNA synthesis had started

caused a 12-hr delay in the onset of synthesis without altering the shape of the DNA synthesis curve, and without altering the time relationship between the peak of synthesis and the beginning of actual mitosis, in fact, there was no further delay in mitosis. This means that DNA synthesis and mitosis were merely postponed for 12 hr and it does not mean that there was no damage to DNA, because a large percentage of these cells coming into division showed chromosome abnormalities (as detected by Professor Koller).

# RADIATION-INDUCED DISTURBANCES OF THE LIPIDS OF CELLULAR MICROSTRUCTURES

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## SUMMARY

The content of different lipid fractions has been determined in cellular microstructures of the rabbit liver and intestinal mucosa under normal conditions and after total exposure to 1,000 r  $\gamma$ -rays of cobalt-60. Determinations were made on summary preparations of cytoplasmic organelles and of hyaloplasm of liver cells 24 and 72 hr after exposure and in mitochondria, microsomes and hyaloplasm of intestinal mucosa cells 2, 24 and 72 hr after exposure. Post-irradiation changes were studied in the following lipid fractions: "free"—extractable by petroleum ether; "loosely-bound"—extractable by methanol-chloroform mixture (minus the "free" lipids); and "firmly-bound"—which can be extracted after alkaline hydrolysis from the residue after methanol-chloroform treatment.

Considerable changes in the content of individual lipid fractions and their quantitative interrelation in various micromorphological cellular components may occur within 2 hr of radiation injury. Later on these changes proceeded in various directions. The radiation-induced changes in the content of the various lipid fractions of sub-cellular components of irradiated intestinal mucosa differ from those of liver. The simultaneous changes of the lipid fractions of microsomes, mitochondria and hyaloplasm may be variously directed.

The changes in the composition of lipid complexes of cellular organelles produced by ionizing radiation may be a cause of many subsequent disturbances in the cellular metabolism of damaged tissues.

Recent advances in cytology enable us to visualize clearly the great role of individual cellular micro- and ultramicrostructures in the biological activity of cells. The present-day achievements of biochemistry have made available many concrete facts characterizing the participation of cell micromorphological components in the metabolic processes and their regulation. The biochemical turnover in cells proceeds in a complex multiphase organized system, and its individual links are localized and intimately connected with definite *structures*. It may be assumed that these structures composed of biochemically active substances are not merely passive plastic formations, but represent spatially oriented participants of many-sided reactions which "endow" them with high-rate arrangement. Heterogeneity of the cell contents

leads, in particular, to the possibility of a counter-directed development of some definite reactions in various cell compartments, within and upon its various structures. Hence, the highly important role of topochemical investigations is quite evident.

It is noteworthy that the functional peculiarities of the cell organelles are determined to a great extent by the lipid-protein complexes which are their components. Therefore, various changes of these complexes as a whole or of their single components, i.e. lipids or proteins, ought to effect the course of biochemical reactions dependent upon such substances (e.g. by the changes of adsorptive bonds with the enzymes and their substrates and also by the changes of the distribution of charges, and likewise by the changes of the distance between active groups).

Naturally, great attention should also be attached to those shifts observed in the lipid-protein complexes of cell organelles which develop as a result of the action of ionizing radiations.

The action of radiation is accompanied by a series of disturbances observed in various biochemical complexes within the cell. First there are changes in the nucleoproteins. In the course of radiation-biochemical investigations the more or less pronounced disintegration of complexes was emphasized.

Sissakian (1955) pointed out that the disturbance in the co-ordination and in the coupling of the enzymatic processes is one of the primary consequences of radiation injury. It may be basically a result of disturbances of the biochemical complexes in the cell microstructures. Kuzin (1955) indicated that irradiation initiated the depolymerization of high-polymer substances constituting such structures. He noted also the disintegration of lipoproteins following irradiation. Possibly various types of alteration in respect of composition and of the dynamics of the chemical components of tissue and their dislocations at the micromorphological level are indispensably involved in the mechanism of the initial and very early effects of radiation.

At our laboratory Ilyina *et al.* (1957) showed that soon after a single X-ray exposure (800 r) rats display a transient reduction of the relative lipoprotein content in mitochondria and microsomes of the liver cells. Blokhina (1959a) later established that, in the course of the development of the radiation disease, a progressive decrease of the lipid content may be observed in the lipoproteins of the liver mitochondria. This evidence pointed also to a certain degree of disintegration of the lipoprotein complexes of the cytoplasmic organelles in the liver cells and to disturbances of their resynthesis leading to an altered composition. According to other data obtained by Blokhina (1959b) the total lipid content of the mitochondria and microsomes showed an initial increase



following irradiation and subsequently decreased; the phospholipid content of the same cellular microstructures dropped slightly; at this point the total lipid content of the hyaloplasm is increased.

With the aim of promoting the study of disturbances initiated by radiation injury in the lipid complexes of cellular organelles and in their lipid metabolism in general, we investigated the quantitative ratio between various lipid fractions of the entire cytoplasm and the hyaloplasm of the liver cells in rabbits exposed to the action of  $\gamma$ -rays of  $^{60}\text{Co}$  at a dose of 1,000 r and a dosage rate of 500 r/min (Blokhtina and Doemin, 1959). This radiation dose is sufficient to give an acute form of the radiation disease in rabbits. Most of the animals died within 5 to 7 days of exposure.

As a rule in every experiment one of two rabbits (of equal weight) was irradiated; the other was used as a control; 16 hr before the experiment both animals were deprived of food and after irradiation both animals were killed with an air embolism. The livers of the rabbits were washed with isotonic saline and homogenized with a ten-fold amount of isotonic sucrose. Cytoplasm and hyaloplasm preparations of the rabbit liver cells were made as described in detail earlier (Ilyina *et al.*, 1957).

The dry weight of the preparations (5 ml) was established after elimination of sucrose by dialysis for 48 hr against distilled water. The contents of the dialysis bag were transferred to a weighing bottle and dried at 60°C to constant weight.

The content of three lipid fractions was measured in the cytoplasm and hyaloplasm preparations. These fractions were called "free", "loosely bound" and "firmly bound" lipids.

The determination of the "free" lipids was made:—10 ml of the cytoplasm or hyaloplasm preparations was shaken with 25 ml of petroleum ether at room temperature for 3 min; after centrifugation at 2,000 r.p.m. for 5 min the top ether layer was decanted into a separate flask. A similar extraction with new portions of ether was repeated 4 times. The combined lipid ether extracts were evaporated at room temperature to minimal volume and the lipid contents determined in an aliquot by a modification of Bloor's technique (1947).

The total lipid content was estimated by utilizing another 10 ml of sample of the cytoplasm or hyaloplasm preparations. At first the lipids were extracted according to the method of Foleh *et al.* (1951). After numerous extractions with the aid of methanol-chloroform mixtures, the residue was subjected to alkali hydrolysis with a 30-fold quantity of 8 per cent alcoholic alkali solution for 40 hr; the fatty acids were extracted from the hydrolysate by Romantzev's method (1952). The content of total lipids was determined by adding the amount of lipids

extracted according to Folch and of the fatty acids released by subsequent alkali hydrolysis (the fraction of the "firmly bound" lipids).

The quantity of the "loosely bound" lipids was ascertained by estimating the difference between the quantity of lipids extracted with the methanol-chloroform mixture and that soluble in petroleum ether ("free" lipids).

The hyaloplasm comprises about 40 per cent of the total dry weight of the liver cell cytoplasm. The approximate data with regard to the content of the lipid fractions in the cytoplasmic microstructures (in round figures) were calculated on the basis of this value and the results of the determination of lipid fractions in the entire cytoplasm and in the hyaloplasm separately.

The determinations were performed on control animals and at 24 and 72 hr following radiation injury; under the conditions of our experiments these times corresponded to the beginning and the highest point of the radiation disease.

Under normal conditions the liver tissue in rabbits is relatively very rich in lipids; the total content of the latter in the cytoplasmic organelles and in the hyaloplasm is approximately equal and comprises about 30 per cent of the dry weight. It was established that under normal conditions according to the ratio between the separate lipid fractions the cytoplasmic organelles and the hyaloplasm of the liver cells differ greatly. In the cytoplasmic organelles all the lipids are actually in a

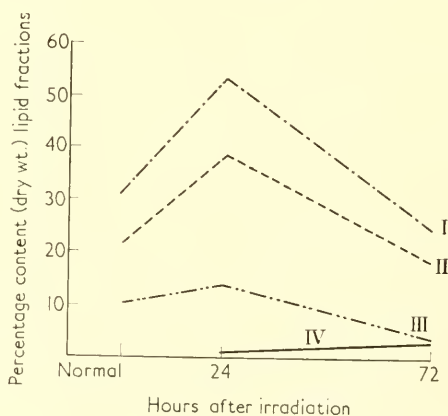


Fig. 1.—The content (percentage of dry weight) of various lipid fractions in the cytoplasmic organelles of the rabbit liver cell under conditions of acute radiation disease.

- I. Total lipid content;
- II. "Loosely bound" lipids;
- III. "Firmly bound" lipids
- IV. "Free" lipids.

bound state, mostly in the "loosely bound" form. The hyaloplasm reveals a rather different picture: about 40 per cent of its lipids are in the "free" state, and the bound lipids are found in the "firmly bound" fraction (Figs. 1 and 2).

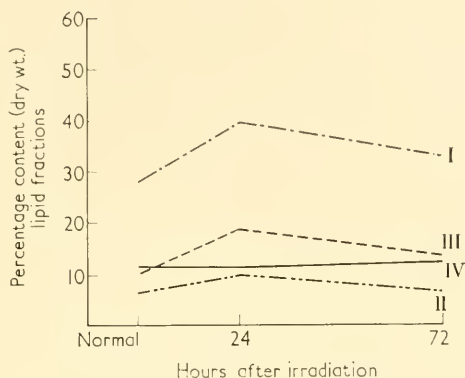


Fig. 2.—The content (percentage of dry weight) of various lipid fractions in the hyaloplasm of rabbit liver cells under conditions of acute radiation disease; curves as in Fig. 1.

Twenty-four hours after irradiation (in accordance with the investigations carried out earlier) an increase in the total lipid content was found especially in the mitochondria and microsomes (Table I). In the hyaloplasm these increases were a consequence of an increase in the bound (mostly "firmly bound") lipids; the portion of the "free" lipids decreased (up to 30 per cent). In the organelles an even greater increase in the content of bound lipids occurred with a pronounced tendency to the accumulation of "loosely bound" lipids. It should be noted that a small (in terms of absolute values), but relatively substantial increase of the "free" lipids up to values surpassing the normal by ten-fold (up to 2 per cent of the total lipid content) was observed.

Seventy-two hours later the ensuing decrease of the total lipids to an apparently almost normal value was accompanied by further disturbances in the ratio between the different lipid fractions. The hyaloplasm showed a considerable reduction of the bound fractions along with an increase of the content of "free" lipids; such a situation led to a certain normalizing of the relations between the lipid fractions. At this point the content of bound lipids in the cytoplasmic organelles dropped below the normal values; and the "firmly bound" fraction was particularly lower in this case. The content of the "free" lipids in cytoplasmic organelles kept increasing and exceeded the normal values

by 30-fold. This resulted in a pronounced qualitative shift of lipid composition in the cytoplasmic organelles.

Possibly at the beginning of the development of the radiation sickness lipoprotein disintegration was retarded both in the cytoplasmic organelles and in the hyaloplasm of the rabbit liver cells; later on this state in the cytoplasmic organelles changes; and an intensified disintegration begins in them (of the more stable components, especially). They also display a reduced capacity for binding "free" lipids. In the hyaloplasm "firmly bound" lipids proved to be the most stable ones.

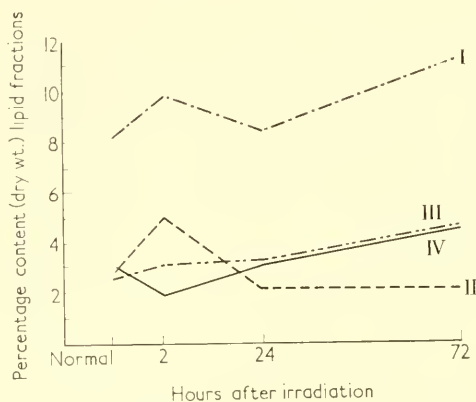


Fig. 3.—The content (percentage of dry weight) of various lipid fractions in the mitochondria of intestinal mucosa cells of rabbits under conditions of acute radiation disease; curves as in Fig. 1.

Similarly, investigations of the intestinal mucosa in rabbit were conducted somewhat later under similar conditions of the radiation action; unlike the previous series these experiments did not deal with preparations of the entire cytoplasm, but with the mitochondria and microsomes separately. The mitochondria and microsome preparations as well as the preparations of the liver cell cytoplasm were produced from homogenates of the intestinal mucosa by differential centrifugation using a modified high-speed centrifuges ASL-1 and ASL-2. Additional determinations were made 2 hr following exposure. The results obtained are shown in Figs. 3 to 5 and Table II.

In total lipid content and that of the separate fractions determined in our experiments, the cells of the intestinal mucosa of the rabbit differ considerably from liver cells. The total lipid content, as per cent dry weight, is significantly less in the intestinal cells.

It is noteworthy that, although under normal conditions the "free"

TABLE I. *The quantitative and qualitative changes in the lipid composition of the cytoplasmic organelles and hyaloplasm of liver cells in rabbits under conditions of acute radiation injury*

Conditions of the experiment	Total lipid, % normal	Free lipids % normal	% total content	Loosely bound % normal	% total content	Firmly bound % normal	% total content
<i>Cytoplasmic Organelles</i>							
Normal	—	—	0.3	—	68	—	32
24 hr following irradiation	+70	+1000	2	+80	72	+40	26
72 hr following irradiation	-20	+2000	12	-15	72	-60	16
<i>Hyaloplasm</i>							
Normal	—	—	39	—	22	—	39
24 hr following irradiation	+43	+5	29	+57	24	+75	47
72 hr following irradiation	+15	+12	37	+12	21	+29	42

TABLE II. *The quantitative and qualitative changes in the lipid composition of the cytoplasmic organelles and hyaloplasm of the cells of the intestinal mucosa in rabbit under conditions of acute radiation injury*

Conditions of the experiment	Total lipid % normal	Free lipids % normal	% total content	Loosely bound % normal	% total content	Firmly bound % normal	% total content
<i>Mitochondria</i>							
Normal	—	—	35	—	34	—	31
2 hr following exposure	+20	—	18	+75	50	+24	32
24 hr following exposure	+2	+7	37	-25	25	+28	38
72 hr following exposure	+39	+62	41	-25	18	+84	40
<i>Microsomes</i>							
Normal	—	—	22	—	29	—	49
2 hr following exposure	+94	+300	46	-7	14	+60	40
24 hr following exposure	-11	+100	50	-54	15	-36	35
72 hr following exposure	+48	+100	30	-29	14	+68	56
<i>Hyaloplasm</i>							
Normal	—	—	45	—	10	—	45
2 hr following exposure	+62	-23	21	+450	30	+77	49
24 hr following exposure	+3	-23	33	-33	7	+39	60
72 hr following exposure	+55	+39	36	± 0	7	+100	58



lipid content of the cytoplasmatic organelles of the liver is extending low, the content of "free" lipid in the mitochondria and microsomes of the intestinal mucosa is high and amounts to 35 and 22 per cent respectively of the total lipid content. On the other hand, the relative content of the lipid fractions in the hyaloplasm in both tissues is very similar.

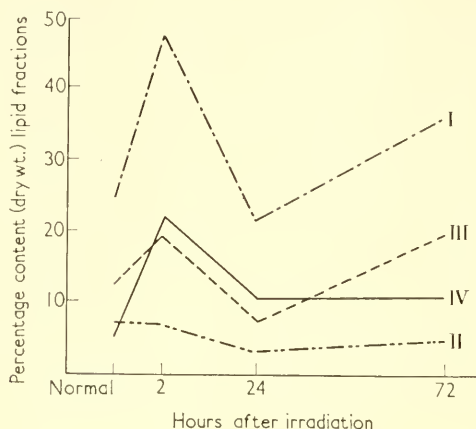


Fig. 4.—The content (percentage of dry weight) of various lipid fractions in cell microsomes of the intestinal mucosa of rabbits under conditions of acute radiation disease: curves as in Fig. 1.

The changes in the total lipid content and its fractions in the cells of the intestinal mucosa following irradiation also differed considerably from the picture observed in the liver. The comparative study of data obtained 24 and 72 hr after irradiation supports this conclusion.

It should be stressed that in the case of the intestinal mucosa the separate investigation of mitochondria and microsomes established that the direction of the changes may differ. The relative content of the "firmly bound" lipids 2 and 24 hr after exposure showed a reduction in the microsomes while in mitochondria there was a tendency for it to increase (72 hr later relative content of the "firmly bound" lipids increased also in the microsomes). As to the content of the "loosely bound" lipids, immediately after exposure it fell considerably in the microsomes; on the contrary in the mitochondria it showed a marked initial increase and a reduction began only later. The relative content of "free" lipids rose significantly in the microsomes, whereas in the mitochondria it showed no difference from the control (it was even halved by the second hour following exposure).

This observation will bring us back again to the liver analysis; the

aim of which is to reveal post-exposure separate alterations in the liver mitochondria and microsomes.

Contrary to the observations for the liver cells, the mitochondria and the microsomes in the small intestinal mucosa showed no progressive reduction in their content of "firmly bound" lipids. Indeed, as noted above, this content had a tendency to increase (especially in the mitochondria). In this case the absolute quantity of this fraction exceeded the control (except the reduction in the microsomal content

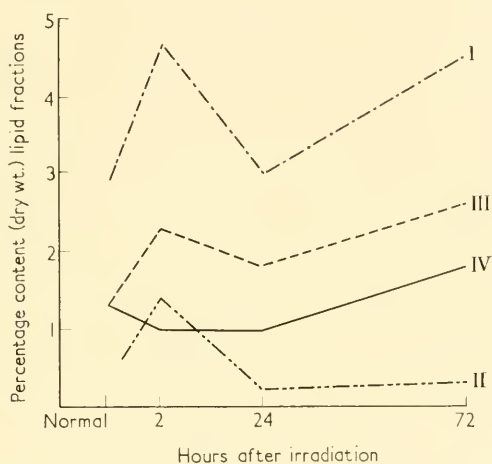


Fig. 5.—The content (percentage of dry weight) of various lipid fractions in the hyaloplasm of the intestinal mucosa cells of rabbits under conditions of acute radiation disease; curves as in Fig. 1.

24 hr after irradiation). The content of the "loosely bound" lipids in the cytoplasmic organelles of the small intestinal mucosa showed a tendency to fall, whereas the content of this fraction in the liver cells was rather stable.

The increase of the total lipid content in the hyaloplasm of the small intestinal mucosa cells, contrary to the data obtained in the liver studies, was accompanied by pronounced changes between the lipid fractions with a reduced amount of "free" lipids and relative increase for the "firmly bound" ones.

Thus, the radiation injury resulted in pronounced disturbances of the lipid composition of the morphological components also of the cells of the intestinal mucosa in rabbit, but these alterations differed from the ones observed in the liver tissue.

It is of interest to note that the considerable shifts in the lipid

fractions of the cell organelles in the small intestinal mucosal cells were observed rather early, i.e. within 2 hr after irradiation.

Undoubtedly, the early and subsequently noted divergence from the norm in the lipid complexes of the cell organelles (both in content and composition) which develop after the irradiation may in turn bring about many alterations in cell metabolism. The oxidative transformations with the transport of electrons along the respiratory chain, oxidative phosphorylation, peptide bond synthesis etc. associated with the activity of mitochondria and microsomes should be stressed first of all.

The results obtained raise a series of questions in the field. A deeper knowledge of the pathological significance of these changes will require a knowledge of the chemical composition of the lipid fractions examined in our experiments. The quantitative and qualitative alterations in the lipids of lipoprotein complexes especially deserve examination. It is important to find ways to solve the most intricate problems dealing with the mechanism responsible for these disturbances and their issue for the biochemical, and the entire biological activity of the cells.

In fact, all these data lead to one common problem of important practical significance; which of these alterations are a true consequence of the destructive effect of ionizing radiation and which of them should be ascribed to compensatory reactions on the part of the organism.

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#### DISCUSSION

BACQ: You compare liver and the mucosa of the small intestine, but the conditions found in these organs following radiation exposure are very different. Liver cells are not destroyed, whereas cells of the intestinal mucosa undergo rapid degeneration. Cell population changes also differ greatly in these organs. In the case of the intestinal mucosa, within 24 hr of exposure you are dealing already with a quite different cell population.

Both in liver and in intestinal mucosa you have observed after exposure an increase of the total lipids, as may be seen from the data you have presented.

But the mechanisms underlying this change in the cells of these two tissues may be different. As for radiation induced changes of the lipid metabolism in liver, I have worked a little in this field, and we are well aware of the fact that in this case an important role could be played not by a direct effect of radiation on the organ, but by an indirect one. Changes of the lipid metabolism in the liver may be ascribed in particular to an increased cortisone secretion found following irradiation. Cortisone is capable of stimulating lipid deposition. I do not know how cortisone would effect different lipid fractions, but its administration raises the total lipid content. The process occurs as a rapid and sharp reaction.

DOEMIN: I would hardly be able to give the answers your remarks call for. Indeed they amount to a question about the causes of the changes we have reported. Up to the present this has not been the purpose of our investigations. Before starting to decipher the mechanisms involved we thought it necessary to gather some pertinent facts, to find out what is changing and how it is changing.

Of course we realize all the differences there are between liver and intestinal mucosa both in cellular composition, and in biochemical peculiarities, as well as the differences of their reactions to radiation. I agree with you that these differences are great indeed. Precisely because of that we have chosen these organs for comparison. And now there arises the problem of elucidating the factors that induce the changes we have established as specific for this or that organ.

In future work biochemical changes in diverse areas of the tissue will be evaluated differentially and compared with the histological picture.

I quite agree with you that on the one hand, metabolic disturbances in certain tissues of the irradiated organism may be an indirect effect of some distant influences (for example hormonal) while on the other hand, the mechanism of the disturbances in the lipid metabolism of the liver following irradiation (among others an increase in the total lipid content) may be quite different from that operating for the intestinal mucosa. Lipid accumulation in the liver may be a result of neogenesis or deposition from the blood, or both. As was shown also in our laboratory† in experiments on angiotomized dogs with cannulated hepatic and portal veins, the intestines in fasting animals following irradiation were in most cases enriching the blood with lipids in spite of hyperlipemia (under normal conditions the reverse was true). At the same time the liver was releasing the lipids into the blood stream in a smaller quantity than usual, or was even taking it up. These phenomena have been registered already within 2 hr of irradiation. It is possible that some of the changes in the content of certain lipid fractions of the liver cell components reported to-day, are related to this "pumping over" of the lipids into the liver from other abdominal organs.

During the studies of different aspects of the acetylcholine metabolism in the body following irradiation, it was shown in our laboratory‡ that immediately after the radiation exposure begins a transport of considerable amount of acetylcholine into the liver by the portal blood. This persists throughout the period of the acute radiation sickness. At the same time it is known§ that acetylcholine can activate phospholipid metabolism. Activation of the metabolism of

† DOEMIN, N. N. (1960). In "Voprosy radiobiologii". Vol. III, p. 158. Leningrad; SMIRNOV, K. V., and SHATERNIKOV, V. A. (1960). *Vopr. med. Khim.* **6**, 464.

‡ SMIRNOVA, K. V. and SHATERNIKOV, V. A. (1960). *C.R. Acad. Sci. U.R.S.S.* **131**, 961.

§ HOKIN, L. E. and HOKIN, M. R. (1955). *Biochim. Biophys. Acta* **16**, 229; (1959). *J. Biol. Chem.* **234**, 1387; and other papers by the same authors.

the lecithins, cephalins, sphingomyelins and inosine phosphatides in the liver was observed in our laboratory† already within the first few hours following irradiation. It is possible that at least some of the radiation-induced changes of the lipid metabolism in the liver are due to disturbances in the acetylcholine metabolism. Later we established‡ that acetylcholine, while inhibiting (*in vitro*) phospholipid metabolism in normal liver slices, activates it (in certain low concentrations) in slices of liver taken from irradiated animals.

However, post-irradiation increase of lipid biosynthesis in the liver (as well as in other organs) due to normal mechanisms is also highly probable.

ARDASHNIKOV: To decipher all these pathways further studies are necessary. What is the statistical reliability of the changes you have observed following irradiation?

DOEMIN: Since the experiments performed were rather time consuming, the number of replicates was not particularly large. For controls a minimum of 10 assays were performed for every point whereas for the post exposure period the number was not less than 4 or 5. Here of course only statistically significant changes were mentioned, and no reference was made to any which were not.

† KAINOVA, A. S. (1960). *Biochimia*, **25**, 540.

‡ DOEMIN, N. N. and KAINOVA, A. S. (1961). *Radiobiologia*, **1**, 192.



# THE SIGNIFICANCE OF FREE DEOXYRIBONUCLEOTIDES IN RADIATION DAMAGE

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## SUMMARY

The authors present a survey of experiments indicating that a part of the radiation inhibition of DNA synthesis in animals may be caused by some interference of radiation with the synthesis of DNA precursors. Supplementation of bone-marrow from irradiated guinea-pigs with nucleotide fractions of chick-embryo extract enhanced the synthesis of DNA. Addition of deoxycytidylic acid to irradiated bone-marrow stimulated the incorporation of  $^{32}\text{P}$ -phosphate and  $^{14}\text{C}$ -formate into reticular cells. Administration of some, especially pyrimidine, deoxyribonucleotides into irradiated mice increased the mitotic index in their bone-marrow and tended to restore to normal the increased ratio of DNA purines/pyrimidines. The content of free deoxyribosides increased in rat tissue after irradiation, but the increase of deoxyribonucleotides in rat liver, which takes place after partial hepatectomy, was reduced after irradiation.

In our earlier experiments, we observed that some deoxynucleotides, especially deoxycytidylic and thymidylic acids, had a favourable effect on the course of radiation disease, when the dose of radiation was sublethal and that of the nucleotide amounted to 0.1 to 1.0 mg per mouse. A more speedy recovery of the WBC count was observed, particularly in the increase of young leucocyte forms, the rate of survival increased on the average by 20 per cent and the mitotic index of the bone-marrow was also higher as compared with the irradiated control. When deoxycytidylic acid in a concentration of about  $30\text{ }\mu\text{g/ml}$  was added to a suspension of bone-marrow from irradiated guinea-pigs surviving *in vitro*, DNA-synthesis increased in comparison with the irradiated control. In these experiments, we proceeded from deliberations based on Jacobson's hypothetical humoral factor of the blood-forming tissue presuming that free deoxyribonucleotides or other compounds related to them might be the factor itself; and, further, that the inhibition of DNA synthesis, as observed after irradiation, was of

secondary character and that the inhibition of synthesis of the DNA-precursors was primary. (Soška *et al.*, 1958; 1959).

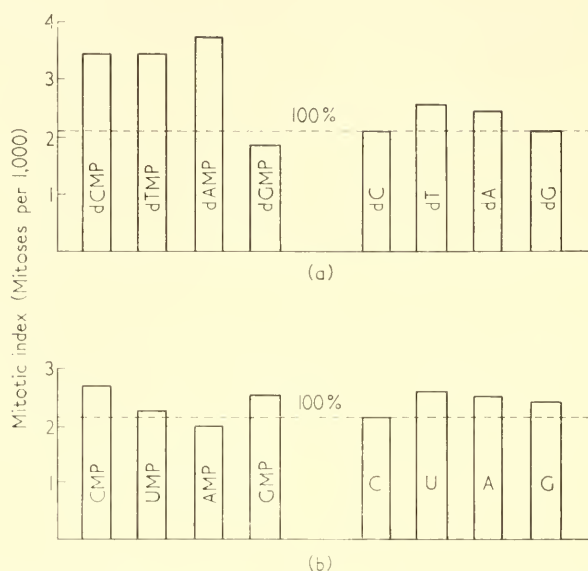


Fig. 1.—The mitotic index in the bone marrow of mice (total-body irradiated with a dose of 600 r) on the sixth day after irradiation (a) and on the fifth day after the administration of nucleic acid derivatives (b). The height of the columns corresponds to the mitotic index, expressed as number of mitoses per 1,000 cells. The horizontal line with the indication 100 per cent corresponds to the mean mitotic index in control irradiated mice, injected with saline only.

The effect of some components of the nucleic acids on the mitotic index of the bone marrow of mice on the sixth day after irradiation with a dose of 500 r was compared. The substances subjected to trial were injected intraperitoneally 24 hr after irradiation in a dose equivalent to 0.3 mg of deoxycytidylic acid. A statistically significant increase of the mitotic index, as compared with the irradiated controls, was found only after the administration of deoxycytidylic, thymidylic and deoxyadenylic acids. (Fig. 1). Deoxyguanylic acid had either no effect at all, or, in one experiment, the mitotic index was decreased after its administration. The other nucleotides and nucleosides had either a slight, not significant effect (cytidylic acid) or no effect whatsoever.

Some substances were then compared according to their effect on DNA synthesis in irradiated bone-marrow. The guinea-pigs were irradiated with a dose of 600 r, a suspension of their bone-marrow was prepared 6 hr later, and incubated with  $^{32}\text{P}$ -phosphate for 6 hr. Then,

TABLE I. *The effects of some fractions of chick-embryo extract on DNA synthesis in bone-marrow suspensions from irradiated guinea-pigs*

Experiment 1. Fraction added	Relative specific activity of DNA-phosphorus:
No extract	100
0.6 N HClO <sub>4</sub> (acid-soluble) extract	112
Acid-soluble extract passed through a column of Dowex 50, H <sup>+</sup> -form, and eluted with water	156
Acid-soluble extract passed through a column of Dowex 2, acetate form, and eluted with water	104
Acid-soluble extract, after adsorption on charcoal and elution with water	92
Experiment 2. Fraction added	Relative specific activity of DNA-thymidylate-phosphorus:
No extract	100
Ringer-solution extract	244
Acid-soluble extract	145
Acid-soluble extract, passed through a column of Dowex 50, H <sup>+</sup> -form and eluted with water	189
Acid-soluble extract, passed through a column of Dowex 2, acetate form and eluted with water	140
Acid-soluble extract, bound by a column of Dowex-2, acetate form, and eluted with 0.5 N HCl, the eluate adsorbed on charcoal and eluted with pyridine, the pyridine distilled off	250
Experiment 3. Fraction added	Relative specific activity of DNA-phosphorus:
No extract	100
Ringer-solution-extract	123
Acid-soluble extract	123
The acid-soluble extract was passed through a column of Dowex 2, Cl <sup>-</sup> -form, the fractions were obtained by elution with water and increasing concentrations of chloride (according to Cohn) adsorbed on charcoal, eluted with pyridine and the pyridine was distilled off under reduced pressure:	
Eluted with water	not tested
Eluted with 0.01N NH <sub>4</sub> Cl	not tested
Eluted with 0.01 N HCl	157
Eluted with 0.01N HCl+0.02 N NaCl	119
Eluted with 0.01 N HCl+0.02 N NaCl	219
Eluted with 1 N HCl	146

The fractions were all adjusted to the same volume equal to that of the original 20 per cent chick embryo-extract and each of them was tested by addition of the same volume to the suspension of irradiated bone-marrow, incubated in the presence of <sup>32</sup>P-phosphate.

the specific activity of bone-marrow DNA-phosphorus was determined. Under these conditions, DNA synthesis is reduced to 30 per cent of the value obtained in non-irradiated bone-marrow. The substances to be tested were added to bone-marrow suspensions in concentrations of 10 to 90  $\mu\text{g/ml}$ . Positive results were achieved with deoxycytidylic acid as well as with thymidylic acid, while deoxycytidine and thymidine, in contradistinction to experiments *in vivo* were also effective, though to a lesser degree than the respective nucleotides (Drášil *et al.*, 1959). The effect of all the above-mentioned substances on non-irradiated marrow was considerably smaller than the effect on irradiated marrow, but, on the other hand, DNA synthesis in non irradiated marrow was increased in the presence of adenosine-triphosphate or cytidylic acid.

Using the same procedures, we concurrently tested the effectiveness of chicken embryo extract. Fresh embryo-extract increased DNA synthesis as much as 86 per cent, while the effect of the extract heated for 3 min to a temperature of 70°C was only slightly lower, no effect being obtained with a 3-week old extract. An acid extract prepared with 0.6 M perchloric acid was also effective. If such an extract had been filtered through charcoal, the filtrate was not effective (Table I). On the other hand, a pyridine eluate from this charcoal was effective too. A fraction of the acid-soluble extract passing through the column of Dowex 50 in H<sup>+</sup>-form was effective, while the effectiveness of the fraction passing through Dowex 2, acetate, at pH 7.0, was reduced. When this anion-exchanger was eluted by HCl the eluate was effective. In the next experiment, the acid embryo extract was subjected to fractionation on Dowex 2—chloride according to Cohn and Carter (1950). The most effective fraction proved to be that corresponding to triphosphates, then to mono- and "tetraphosphates". In this experiment, the effectiveness of the whole acid-soluble extract did not differ from that of the extract in Ringer solution.

Several experiments with bone-marrow were carried out by autoradiography. After incubation with <sup>32</sup>P-phosphate or <sup>14</sup>C-formate and eventually with a nucleotide, the bone-marrow suspension was washed with Hanks' solution, then with acetic acid and smears were prepared. After exposure with a sensitive emulsion and staining with MGG-stain, the number of cells that had significantly more grains than the corresponding area of the background was determined. In the presence of deoxycytidylic acid, the incorporation of <sup>32</sup>P-phosphate into the reticular cells increased 2.5 times and 3 times in non-irradiated and irradiated marrow respectively (Table II). Control experiments carried out concurrently showed that under the same conditions two thirds of the incorporation of <sup>32</sup>P into the bone-marrow could be accounted for

TABLE II. *The effect of dCMP  $^{32}\text{P}$ -phosphate and  $^{14}\text{C}$ -formate incorporation into bone-marrow cells of irradiated and non-irradiated guinea-pigs studied by autoradiography.*

Type of cell	$^{32}\text{P}$ -phosphate incorporation				$^{14}\text{C}$ -formate incorporation			
	non-irradiated		irradiated		non-irradiated		irradiated	
	control	dCMP	control	dCMP	control	dCMP	control	dCMP
All cells	37.6	48.0	23.3	29.0	35.8	41.4	24.6	25.4
Reticular cells	12.3	30.9	4.7	13.9	9.4	31.3	4.5	9.9
Myeloblasts and proerythroblasts	70.9	61.5	35.0	33.3	73.5	57.5	30.7	30.0
Erythroblasts	91.5	96.4	75.7	82.8	83.2	95.9	74.4	84.3
Promyelocytes	47.9	44.5	24.2	17.1	64.3	51.9	22.6	22.6
Myelocytes	51.0	51.8	39.3	41.2	46.3	42.5	32.5	34.8

Mean numbers of cells showing significantly higher grain counts than the background, expressed per hundred cells of that cell type.

by incorporation into DNA. Similarly, the incorporation of formate, which is to a considerable degree a specific precursor of DNA-thymine in bone-marrow *in vitro*, indicated a threefold and two-fold increase of DNA-synthesis in non-irradiated and irradiated reticular cells respectively. The results thus show that the increased DNA-synthesis in the presence of deoxycytidylic acid is caused for a great part by increased incorporation into the reticular cells. At the same time, the presence of free deoxycytidylic acid may be a limiting factor for DNA-synthesis in the reticular cells of bone-marrow.

Under similar conditions, the effect of thymidylate was also studied.  $^{32}\text{P}$ -phosphate incorporation into DNA, determined in the entire bone-marrow suspension after fractionation according to Schmidt and Thannhauser without autoradiography has indicated increased DNA-synthesis in the presence of thymidylate. Formate incorporation was reduced at the same time, which reflects the dilution of the newly synthesized  $^{14}\text{C}$ -thymidylate by added thymidylate. The first results obtained by autoradiography have also shown increased  $^{32}\text{P}$  incorporation into irradiated reticular and "blast" cells in the presence of thymidylate, but paradoxically they have also revealed an increase of formate incorporation into both irradiated and non-irradiated reticular cells. In "blast" cells, the expected reduction of formate incorporation was found.

Deoxycytidylic and thymidylic acid had also an effect on the purine/pyrimidine ratio in the spleen of irradiated rats (Paleček and Soška, 1960). This ratio is increased to more than 1 on the fifth day after irra-



diation (Berenbom and Peters, 1956). The injection of deoxycytidylic acid after irradiation has modified the guanine/cytosine and adenine/thymine ratios towards the normal value, while thymidylic acid modified only the ratio G/C and not A/T (Fig. 2).

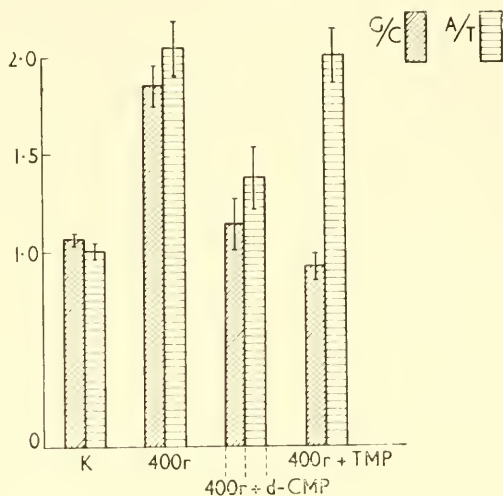


FIG. 2.—The molar ratio of guanine/cytosine and adenine/thymine in the spleen DNA of rats, on the fifth day after irradiation with a dose of 400 r on the fourth day after administration of 1 mg deoxycytidylic or thymidylic acid. The height of the columns corresponds to the molar ratio, standard deviations of the means are also indicated. K  $\equiv$  control, non-irradiated rats.

If the mechanism of the recovery effect of deoxynucleotides or of the embryo extract lies in supplying the missing DNA-precursors, it was expected that after irradiation the content of free deoxyribonucleotides would decrease in the tissue. We carried out the first experimental series with spleens of rats irradiated with a dose of 600 r. However, in the first hours after irradiation no decrease of deoxynucleotides was observed in the tissue (Soška and Sošková, 1959). On the contrary, their great increase was noticed. Only from about the 24th hr following irradiation did the content of these substances fall below the initial level. The level of free deoxyribosides rose somewhat more slowly and the following decrease was also slower. The interpretation of results obtained with the spleen is, however, complicated by the fact that the cell population of this tissue is not homogeneous, that the contribution of the particular cell types changes after irradiation, and the fact of extensive cell death.

For this reason, regenerating rat liver was used for our next experiments. This material has the advantage that its cell population is

relatively homogeneous and does not change after irradiation. According to Holmes (1956) DNA-synthesis progresses most intensively 24 hr after partial hepatectomy and the inception of the synthetic period is delayed by 12 hr by irradiation in the presynthetic period.

The deoxyribosides were detected by the microbiological method of Hoff-Jorgensen (1952), the nucleotides were separated from nucleosides on a column of strong anion-exchanger at pH 7.0. Nucleotides were converted into nucleosides by means of an enzymatic preparation from snake venom before microbiological determinations.

In the first experiment, the rats were irradiated with a dose of 600 r 1 hr after partial hepatectomy. The deoxyribose compounds were determined 25 hr following hepatectomy. Four groups of rats were investigated concurrently viz. hepatectomized irradiated rats, only hepatectomized rats, only irradiated rats and control rats. Irradiation and hepatectomy resulted in an increase in the level of free deoxyribosides (Table III). The highest level was observed in irradiated hepatectomized rats. On the other hand, the deoxynucleotide content was highest in non-irradiated hepatectomized rats. The deoxyribonucleotide content was the lowest in the control rats. Irradiation itself thus resulted in a certain rise of free deoxyribonucleotides, but the further rise produced by hepatectomy was blocked by irradiation.

TABLE III. *The effects of partial hepatectomy and of total-body irradiation on the content of free deoxyribosides and deoxyribotides in liver*

Group	No. of animals	Deoxynucleosides ( $\mu\text{g/g}$ tissue)	Deoxynucleotides ( $\mu\text{g/g}$ tissue)
Control	7	$5.49 \pm 0.34$	$1.44 \pm 0.41$
Irradiated	9	$6.5 \pm 0.43$	$4.55 \pm 0.25$
Hepatectomized	6	$7.35 \pm 0.41$	$9.55 \pm 1.80$
Hepatectomized + irradiated	9	$8.40 \pm 0.68$	$5.60 \pm 0.60$

The irradiation followed 1 hr after hepatectomy, the rats were killed 25 hr after hepatectomy and/or 24 hr after irradiation.

In the next similar experiment (Table IV), the rats were already irradiated 24 hr before hepatectomy. In this experiment, the nucleotides were not separated from nucleosides and all deoxynucleotidic compounds were determined together after enzymatic conversion to deoxynucleosides. The total level of all deoxyribosidic substances in the individual experimental and control groups was similar to that in the preceding experiment. It seems thus, that deoxyribotide synthesis

TABLE IV. *The effect of partial hepatectomy and of totalbody irradiation on the content of free deoxyribosides + deoxyribotides in liver*

Group	No. of animals	Deoxynucleosides + deoxynucleotides ( $\mu\text{g g tissue}$ )
Control	9	$8.7 \pm 0.74$
Irradiated	10	$12.1 \pm 1.05$
Hepatectomized	10	$14.7 \pm 1.08$
Hepatectomized + irradiated	8	$11.2 \pm 0.51$

The hepatectomy followed 24 hr after irradiation, the rats were killed 48 hr after irradiation and/or 24 hr after hepatectomy.

following partial hepatectomy is delayed even by irradiation before hepatectomy.

In the third experiment (Table V), the rats were irradiated 2 hr after hepatectomy and sacrificed later, 28 hr following hepatectomy. The difference in the level of free deoxyribonucleotides in the hepatectomized irradiated and hepatectomized non-irradiated rat livers was still higher than in the preceding experiments, which again indicates a lack of DNA-precursors after irradiation. The differences in the content of polymerized DNA in the respective groups of rat-livers were small (Table V, column 5). At the same time, the content of free "ribonucleotides + ribonucleosides" (all acid-soluble material, absorbing light at  $260 \text{ m}\mu$ ) was almost constant in all groups (column 6) and it was only the "ribonucleoside" content (column 7); (the part of this material, passing a Dowex 1 column, acetate form, at pH 7.0) that changed more conspicuously. These results agree in general with those of Jaffe *et al.* (1959).

An attempt was made to find the differences in the content of the individual substances by means of paper chromatography. In all experimental groups, a substance was found in the liver, corresponding by mobility in butanol-ammonia system and by microbiological activity to deoxycytidine, in accordance with the results of Schneider and Brownell (1957). Purine deoxynucleotides were not found in any group. But in the livers of non-irradiated rats, a distinct spot of an additional deoxyriboside was found. So far it is only known that this substance contains a pyrimidine, but it is probably neither deoxyuridine nor thymidine, but most probably deoxymethyleytidine.

Deoxyribonucleotides were chromatographed in the isobutyric acid-ammonia system. Because of the complex pattern of deoxynucleotides detected on the chromatogram, the individual substances could not be identified. It could only be seen that the number and intensity of the

TABLE V. *The effects of partial hepatectomy and of total body irradiation on the content of free deoxyribosides and deoxyribotides, of DNA and of acid-soluble u.v.-absorbing substances in liver*

Group	No. of animals	Deoxynucleosides ( $\mu\text{g/g}$ tissue)	Deoxynucleotides ( $\mu\text{g/g}$ tissue)	DNA (mg/g tissue)	Total u.v.-absorption (/g tissue)	"Nucleosides" u.v.-absorption (/g tissue)
Control	9	$8.85 \pm 0.35$	$0.95 \pm 0.51$	$2.26 \pm 0.13$	$104.0 \pm 1.6$	$5.95 \pm 0.24$
Irradiated	10	$9.85 \pm 0.16$	$2.94 \pm 0.34$	$2.13 \pm 0.11$	$102.0 \pm 2.25$	$9.85 \pm 0.52$
Hepatectomized	10	$9.19 \pm 0.53$	$16.8 \pm 1.8$	$2.46 \pm 0.06$	$103.9 \pm 2.0$	$9.12 \pm 0.55$
Hepatectomized + irradiated	10	$10.4 \pm 0.61$	$7.37 \pm 0.49$	$2.09 \pm 0.07$	$101.6 \pm 2.7$	$8.31 \pm 0.53$

The irradiation followed 2 hr after hepatectomy, the rats were killed 28 hr after hepatectomy and/or 26 hr after irradiation.

spots containing deoxyribosidic compounds increased in the sequence from non-irradiated control to irradiated control, irradiated hepatectomized to hepatectomized non-irradiated livers. Since Sugino and Potter (1960) have recently established that the activity of deoxycytidylate-deaminase is reduced after irradiation, it seemed probable that the nucleotides accumulating after irradiation are derivatives of deoxycytidine whereas the others appearing after hepatectomy in non-irradiated animals might perhaps be derivatives of deoxyuridine and thymidine. The nucleotides were dephosphorylated by a snake-venom preparation and subjected then to paper chromatography as nucleosides: preliminary results have, however, indicated the presence of deoxyuridine and thymidine derivatives in all groups except in non-irradiated controls, i.e. also in irradiated groups. The interval of 28 hr after hepatectomy was perhaps too long and the appearance of thymidine derivatives may be the result of recovery.

Summing up, we should like to stress some of the results. The effect of deoxynucleotides on mitosis, the possibility of increasing radiation-inhibited DNA-synthesis by means of deoxynucleotides or by the nucleotide fraction of embryo-extract, further the changes of the content of free deoxynucleotides and deoxynucleosides in regenerating liver after irradiation. They suggest that the effect of radiation on processes leading to DNA-synthesis in animals may be more important than the effect on the process of polymerization or on the macromolecular DNA itself.

This is in accordance with the results of Sugino and Potter (1960) who have found that radiation blocks the enzymatic reactions that result in the synthesis of thymidylic acid. Even then there remains the unsolved question why these enzymes are inactivated, since in the case of other enzymes, no inactivation as a result of irradiation *in vivo* has been so far observed. Quite on the contrary, a raised enzymatic activity has been often noted. Consequently, it appears that the second possibility is more probable, that is to say that after irradiation there takes place disintegration of large structural subcellular units or entire specialized cells that are carriers of the respective synthetic activity. Experiments with regenerating liver would support the interpretation that irradiation blocks some inductive process resulting in the synthesis of enzymes that make themselves apparent in the preparation of DNA-synthesis, in particular in the synthesis of some deoxyribotides.

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#### DISCUSSION

MANOILOV: Did you administer nucleotides isolated from different organs and tissues or are the phenomena observed characteristic for spleen and bone-marrow alone?

Did the deoxyribonucleotides administered subcutaneously incorporate into spleen DNA as such, or did they undergo preliminary splitting up?

soška: The effect of deoxyribonucleotides on bone-marrow alone has been studied. We have not studied as yet, whether the nucleotides administered are broken down in tissues or not.

UTKIN: How did you determine the mitotic index?

soška: A smear of bone-marrow from the sacrificed mouse was prepared and stained according to Feulgen. The mitotic index was determined in a count of 8,000 to 10,000 nucleated cells. The proportion of the individual mitotic phases was recorded. Dr. Karpfel found that, following exposure, the ratio of metaphases to prophase was changed. If, in the unirradiated mice, the ratio of metaphases to prophase was 0.56, in animals irradiated with 500 r this ratio rose to 1.0 or 1.5. We found that administration of pyrimidine deoxyribonucleotides following exposure brought changes in this picture, the ratio of metaphases to prophase returning partially to normal.



# BIOCHIMIE ET RADIOBIOLOGIE DU NOYAU CELLULAIRE

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## RÉSUMÉ

Les radiolésions du noyau cellulaire sont envisagées du point de vue biochimique. Certaines fonctions du noyau sont particulièrement sensibles: les phosphorylations (Creasy et Stocken, 1958) et la stabilité des ions  $\text{Na}^+$  et  $\text{K}^+$  (Creasy, 1960) sont fortement altérées par des doses de l'ordre de 100 rad. Comme ces ions jouent un rôle important dans la fixation des petits nucléotides dans le noyau, ces déséquilibres produits par l'irradiation pourraient être liés aux effets des rayons X sur le métabolisme des acides nucléiques. Quand on étudie l'incorporation du  $^{32}\text{P}$  dans l'acide désoxyribonucléique du thymus on observe, que pour des doses croissantes, l'inhibition augmente d'abord rapidement, puis plus lentement (Ord et Stocken, 1958). Il en est de même quand on étudie l'incorporation de l'adénine dans l'acide ribonucléique des noyaux isolés (Logan *et al.*, 1959), ou l'incorporation de la phénylalanine dans les protéines. Dans les trois types de métabolisme, on observe que des doses dépassant 300 rad n'augmentent que très peu l'effet observé. Il est encore prématuré d'interpréter ces résultats en fonction des effets biochimiques décrits sur la cellule entière ou en fonction des effets cytogénétiques.

Un grand nombre de données expérimentales indiquent clairement que les radiolésions nucléaires ont des conséquences plus graves pour la cellule que les radiolésions cytoplasmiques. Celles-ci semblent en effet être susceptibles d'être restaurées dans une certaine mesure à condition qu'existe un noyau suffisamment fonctionnel (Errera *et al.*, 1959). Il est donc logique d'essayer de comprendre les mécanismes physicochimiques ou biochimiques des radiolésions nucléaires que l'on commence à connaître du point de vue cytologique. C'est pourquoi il nous a paru intéressant de rassembler dans cette brève communication les données existantes concernant les processus biochimique normaux qui se déroulent au sein des noyaux et la manière dont ceux-ci sont affectés par l'irradiation; nos propres observations seront intégrées à l'image plus générale que l'on peut dégager de la littérature.

## PROCESSUS BIOCHIMIQUES NORMAUX

On sait qu'au cours du cycle cellulaire normal la division est pré-

éédée d'importants phénomènes de synthèse dont ceux qui concernent l'acide désoxyribonucléique (ADN) sont certainement les mieux connus. Mais à côté de l'ADN les chromosomes contiennent des acides ribonucléiques, des protéines, des phospholipides, et il est vraisemblable que ceux-ci se synthétisent au niveau ou dans le voisinage immédiat des chromosomes. D'autre part les nucléoles, riches en acide ribonucléique, en protéines, et en phospholipides se reforment après chaque mitose, et il est vraisemblable également que les organisateurs nucléolaires des chromosomes sont le siège de synthèses actives. D'ailleurs de nombreux travaux utilisant les techniques cytochimiques quantitatives (autoradiographie, microspectrophotométrie) montrent clairement que des précurseurs d'acides ribo- et désoxyribonucléiques, de même que des précurseurs de protéines s'incorporent dans ces structures cellulaires et témoignent vraisemblablement de l'existence de synthèses nettes d'acides nucléiques et des protéines (voir Brachet, 1957). D'un point de vue biochimique plus précis ces mécanismes de synthèses sont moins bien connus, mais ils impliquent vraisemblablement l'existence de systèmes enzymatiques particuliers, soit fournissant l'énergie indispensable soit effectuant les synthèses elles-mêmes. Nous nous préoccupons principalement des systèmes enzymatiques des noyaux de cellules lymphoïdes ou de foie de mammifère, tout en mentionnant à l'occasion ce que l'on sait pour d'autres organes ou même d'autres types d'organismes. Nous ne nous étendrons pas sur ce que l'on sait ces sources nucléaires d'énergie et sur la synthèse de l'ADN, des aspects du problème ayant déjà été envisagés dans d'autres communications.

#### SYNTHÈSE DES ACIDES RIBONUCLÉIQUES

Il semble de plus en plus certain que la grande majorité de l'ARN des cellules qui se multiplient est synthétisé dans le noyau cellulaire: il a même pu être démontré dans les cellules de He-La (Perry, 1960; Perry, Hell et Errera, 1960) qu'environ 60 pour cent de l'ARN cytoplasmique provient du nucléole, et qu'environ 30 pour cent provient du reste du noyau. Il n'est même pas certain qu'il existe de *synthèse cytoplasmique* d'ARN dans les cellules qui se multiplient; d'où l'importance de rechercher dans le noyau cellulaire des radio-lésions qui déterminant des altérations du métabolisme de l'ARN.

On connaît encore mal les mécanismes biochimiques de la synthèse de l'ARN des cellules animales. Chez les microorganismes, par contre, Grunberg Manago et Ochoa ont isolé un système enzymatique qui utilise des nucléotides diphosphates. Cependant, l'étude de divers

tissus animaux, bien que beaucoup moins avancée que celui des micro-organismes, indique l'existence de certains mécanismes de synthèse utilisant les ribonucléosides triphosphates (Cannelakis et Herbert, 1960), mais dans ce cas il ne se fixe que de l'acide cytidylique et adénylique en fin d'une chaîne d'acide ribonucléique de poids moléculaire relativement faible et qui jouerait un rôle important dans le transfert des acides aminés. Un système capable de synthétiser de l'acide polyadénylique pur à partir de l'ATP a été décrit par Edmons et Abrams (1960) dans les noyaux des thymocytes.

Nous sommes, donc, malheureusement loin de connaître les mécanismes de la synthèse de l'ARN, et rien ne nous permet de prévoir que l'ARN des chromosomes et des nucléoles serait synthétisé selon le même mécanisme.

De nombreuses observations, moins directes toutefois, ont été effectuées sur des noyaux isolés. Logan (1957) a, par exemple, observé que l'adénine s'incorpore beaucoup plus rapidement et sans phase de latence dans la fraction d'ARN de noyaux des thymocytes, soluble dans du phosphate 0,1 M à pH 7,0, alors que la fraction insoluble n'incorpore que beaucoup plus lentement et après une certaine latence. Cette fraction qui incorpore rapidement est constituée de particules ribonucléoprotéiques de 25 Å de diamètre dont la localisation dans le noyau n'a pas encore été établie avec certitude (Frenster, *et al.* 1960).

L'incorporation de l'adénine dans l'ARN des noyaux de thymocytes *in vitro* est notablement diminuée par l'irradiation et des expériences radioautographiques sur des noyaux de thymocytes de veau montrent qu'on atteint déjà un effet maximum (environ 30 pour cent d'inhibition) après des doses de 50 rad. Le travail a été repris sur des noyaux de thymus de jeunes rats, et les méthodes biochimiques cette fois, ont montré que la diminution de l'incorporation qui dépasse rarement 20 pour cent chez le rat est déjà maximum après 300 rad et n'augmente pas après des irradiations plus prolongées. Des fractionnements de l'ARN selon la technique utilisé par Allfrey et Mirsky (1958) ont révélé au cours d'expériences préliminaires que l'ARN soluble en milieu salin dilué (ARN I) conserve une activité normale, tandis que la fraction qui n'est pas extraite dans ce milieu (ARN II) incorpore moins d'adénine que celle des cellules non irradiées (Faurès et Errera, sous presse). Il serait tentant de penser qu'il s'agit là d'une fraction d'ARN dépendant plus étroitement de l'ADN et des phosphorylations nucléaires, puisqu'ils sont inhibés simultanément.

Dans le cas du foie de rat, l'incorporation de l'adénine dans les noyaux isolés est sensiblement accrue d'une part par l'addition des microsomes, et d'autre part 48 ou 72 heures après une hépatectomie



partielle. Le rôle des microsomes dans ce processus n'est pas élucidé, mais ils pourraient intervenir dans la conversion de l'adénine en un précurseur mieux assimilable, ou en fournissant d'autres nucléotides qui pourraient être nécessaire à la formation de l'ARN. Les mécanismes de synthèse de l'ARN nucléaire offre certainement un vaste champ d'investigation.

### *Synthèses de protéines*

On ignore encore tout des mécanismes de la synthèse des protéines dans le noyau cellulaire; ce que l'on connaît des systèmes purifiés de synthèses protéiques s'applique à des fractions subcellulaires que l'on a pas encore localisé avec certitude dans les cellules vivantes. Il semble toutefois, d'après des expériences radioautographiques que dans des cellules en croissance la synthèse des protéines se passe de manière indépendante dans le noyau et dans le cytoplasme, et ces données ne font que confirmer celles de Brachet (1957) qui a démontré une synthèse nette des protéines dans des fragments anucléés d'*Acetabularia mediterranea*. Les travaux sur les noyaux de thymus isolés (Mirsky *et al.*, 1956; Allfrey *et al.*, 1957) ont montré d'une part qu'un acide aminé marqué, incorporé dans les protéines des noyaux, n'est pas déplacé si les noyaux sont ensuite mis en présence d'un excès de l'acide aminé non marqué, ce qui indique probablement un phénomène de synthèse. D'autre part Allfrey (1959) a trouvé dans des préparations nucléaires, des systèmes enzymatiques d'activation et de transfert d'acides aminés en beaucoup de points semblable à ceux décrit par Zamecnik *et al.* (1958), dans le cas d'extraits tissulaires (ARN soluble, ARN des ribosomes).

Les mesures d'incorporation d'acides aminés dans les protéines des noyaux semblent donc réellement représenter un métabolisme protéique réel. Toutefois, un point obscur reste à comprendre, ces incorporations d'acides aminés ne sont pas inhibées par la fluorophényl-alanine ou l'éthionine comme le sont la plupart des synthèses protéiques. (Allfrey *et al.*, 1957.) Mirsky, Osawa et Allfrey (1956) ont montré que l'incorporation des acides aminés dans les noyaux cellulaires dépendent des phosphorylations nucléaires, puisqu'on peut les bloquer par l'addition de cyanure, de nitrure, de dinitrophénol, mais pas par le bleu de méthylène qui inhibe les phosphorylations des mitochondries seulement (Osawa, Allfrey, et Mirsky, 1957). Cependant le dicoumarol et le vert de Janus qui n'inhibent *pas* les phosphorylations nucléaires, inhibent l'incorporation des acides aminés. Les phosphorylations nucléaires semblent donc jouer un rôle dans le métabolisme des protéines nucléaires mais ils pourraient ne pas être la source exclusive d'énergie pour ces processus.

D'autre part il semble y avoir un lien assez étroit entre l'incorporation d'acides aminés et le métabolisme ribonucléique puisque le 5:6:di-chlorobenzimidazol inhibe l'incorporation d'acides aminés si cet agent est ajouté en même temps que l'acide aminé.

Fieq et Errera (1958, 1959) et Logan *et al.* (1959) ont montré que l'incorporation de la phénylalanine dans les noyaux de thymocytes de rat est moins modifiée pendant les 30 premières minutes d'incubation après irradiation *in vitro* (50 à 900 rad) que par la suite. Il en est de même pour les noyaux du foie de rat. Dans ce dernier cas, Logan *et al.* ont montré que des mitochondries ajoutées aux noyaux augmentent l'incorporation de la phénylalanine; il a aussi été montré que si on irradie ces mitochondries avant de les additionner aux noyaux, on obtient également une inhibition de l'incorporation des acides aminés.

En ce qui concerne les relations doses-effets étudiées sur des noyaux de thymus de rat, elles suivent une modalité très semblable à ce qui avait été observé dans le cas du métabolisme de l'adénine: inhibition maxima de l'ordre de 20 pour cent pour des doses de 300 rad. Il est intéressant de noter que l'effet d'inhibition par les rayons X peut être partiellement restauré si les préparations des noyaux sont incubées en présence d'acide désoxyribonucléique même dénaturé par chauffage. On se souvient que Mirsky *et al.* (1956) avaient observé une restauration par l'ADN du métabolisme des acides aminés, inhibé par la désoxyribonucléase. Cet effet n'est pas spécifique de l'ADN et semble être en relation avec l'équilibre ionique des noyaux.

#### RÔLE DE L'ÉQUILIBRE IONIQUE DANS LE MÉTABOLISME DES NOYAUX DE THYMOCYTES

Mirsky *et al.* (1956) et Osawa *et al.* (1957) ont montré d'une part que si on traite une suspension de noyaux au moyen d'acétate à pH 5,1, on libère du potassium et des nucléotides en même temps qu'on inhibe l'incorporation des acides aminés dans les protéines. D'autre part, il existe une concentration optimum de  $\text{Na}^+$  pour l'incorporation des acides aminés alors qu'un excès de  $\text{K}^+$  est inhibiteur. Le rapport  $\text{NaCl/KCl}$  et la présence de nucléotides variés joue donc un rôle important dans les phosphorylations nucléaires dont dépend l'incorporation des acides aminés dans les protéines du noyau. Il est donc intéressant que Creasy (1960) ait observé que de faibles doses de rayons X libèrent du sodium des noyaux de la rate de rats (pas de seuil et 80 pour cent de libération après 50 rad) alors que la libération de  $\text{K}^+$  ne débute qu'après 30 rad pour être quasi totale vers 100 rad. Ces doses nous l'avons

vu sont suffisantes pour inhiber les phosphorylations nucléaires et il est vraisemblable que la perte des ions et peut être celle des oligonucléotides en sont la cause. La perte de  $K^+$  et de nucléotides liées aux noyaux peuvent aussi être à l'origine de la diminution de l'incorporation des acides aminés (Osawa *et al.* 1957). Le fait qu'il n'y ait qu'une petite partie des systèmes nucléaires responsables de l'incorporation des acides aminés et des bases azotées qui soit diminuée après de petites doses d'irradiation et qu'une irradiation plus importante n'augmente pas l'effet semblerait indiquer l'existence d'une réserve de dérivés phosphorylés d'origine nucléaire. On devrait toutefois aussi envisager la contamination des noyaux par une quantité suffisante de mitochondries qui pourraient fournir les dérivés phosphorylés nécessaires. On pourrait aussi supposer qu'il existe, comme nous l'avons fait précédemment pour le métabolisme de l'ARN, une fraction protéique plus étroitement dépendante des phosphorylations nucléaires et aussi plus radiosensible.

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#### DISCUSSION

HUG: You were speaking of doses of 200 to 300 r. Is it possible that, at a different dose rate, the effect would be greater, even if small doses were given?

ERRERA: It should be tested. I have not done it.

HUG: Where in your opinion does the disturbance of the RNA synthesis occur?

ERRERA: My first impression is that there are two mechanisms operating: RNA synthesis may proceed in the microsomes or in the nucleoli. But we failed to differentiate them. We tried, but did not succeed in finding a difference between the corresponding nucleoli and microsome fractions. They were difficult to differentiate.

MARCOVICH: RNA is first synthesized in the nucleus and then it comes out into the cytoplasm?

ERRERA: The transfer of RNA from nucleus into cytoplasm was demonstrated by experiments with short and long term incubation of the cells.

We believe that RNA is synthesized in the nucleus and that this is common to all dividing cells. It is the accepted view on the matter until new data appear.

GRAY: I should very much like to know the methods for specific inhibition. It seems to me you have made it clear enough that radiation damage to the chromosomes is dependent in some measure on total phosphorylation. It would be very interesting to study whether it is cytoplasmic or nuclear phosphorylation. Can they be differentiated? In what way is inhibition of phosphorylation connected with mitochondria?

ERRERA: Inhibition of phosphorylation in the nuclei proceeds without affecting the mitochondria. Phosphorylation in the nucleus and cytoplasm can be inhibited separately.

PASSYNSKY: Is it possible to regard the post-irradiation loss of  $\text{Na}^+$  and  $\text{K}^+$  ions as a result of damage to the nuclear membrane? What is the absolute quantity of  $\text{Na}^+$  and  $\text{K}^+$  ions which leaked out from the nucleus following exposure, and what is its quantitative relationship to the ionization value of the medium?

ERRERA: Little is known about this. It is possible that basic proteins are capable of binding positive ions. In the case of  $\text{Na}^+$  about 80 per cent is lost upon exposure to 50 r, whereas in the case of  $\text{K}^+$  there is no loss of ions up to doses of 30 r. When this dose is exceeded, the loss proceeds very rapidly; at 100 r the loss of this ion is almost complete.

PASSYNSKY: If this constitutes damage, I should like to draw attention to the possibility of using impedance measurement techniques to study it.

ERRERA: I have no experience in this field.

PASSYNSKY: As far as I know, the loss of ions may be caused by a damage to the membrane or changes of the absorption. It is a problem which may be studied by means of the impedance technique. If the membrane is damaged, several centres of damage may be discovered.

ERRERA: To elucidate this question, electron microscope pictures should be available, otherwise it would be difficult to get the answer. I have never taken electron micrographs of such nuclei. My belief is that the membrane is severely damaged, but I am not sure of that. For final confirmation good electron micrographs are necessary.

BACQ: In the first experiment you cited you irradiated intact cells and studied synthesis of the RNA and proteins by means of autoradiography. In other experiments it was isolated nuclei you studied?

ERRERA: On the picture given here results of the experiments using whole cells were presented. After that isolated nuclei were used.

KUZIN: If I understood you correctly, in the experiments with isolated nuclei you found approximately 30 per cent inhibition: further increase in the irradiation dose produced no increase in the effect studied. At the same time using whole cells and tissues one may obtain considerably more inhibition. Do you not think that the difference is accounted for by the role played by the cytoplasmic elements?

ERRERA: I am not sure whether we realize what is occurring in the nucleus. But it is a fact that, for protein synthesis, inhibition in intact cells occurs in 90 min. The purpose of this study was to determine the most sensitive process.

MOUTON: May it be that the changes you have observed are caused by the changes in the pH of the medium brought about by irradiation?

ERRERA: I do not think so, since strongly buffered solutions were used throughout the experiments.

MOUTON: It seems possible that there may be a connection between the radiation-induced breakdown and acid autolysis.

HOLLAENDER: You spoke about inhibition of the synthesis of the nucleic acids and proteins. How is this related to mitosis?

ERRERA: Under normal conditions the mitotic index amounts to 5 to 7. It seems to me that the nuclei we studied should be considered as being in a resting phase, not in mitosis.

TOBIAS: You said that RNA synthesis is inhibited by 30 per cent. How is this to be understood? Does it mean that 30 per cent of the nuclei are not taking part in the synthesis, or that synthesis in all the nuclei is inhibited by 30 per cent?

ERRERA: Autoradiography has shown that all the nuclei have been affected equally.



# ON THE MECHANISM OF LETHAL ACTION OF X-RAYS IN *ESCHERICHIA COLI* K12

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## SUMMARY

Crosses have been utilized in order to elucidate the mechanism of the lethal effect of X-rays on *Escherichia coli* K12. These bacteria possess two to three nuclear bodies and are killed according to a one-hit dose-effect relationship. The experiments were designed to search for the existence of X-ray dominant lethal mutations in one of the nuclei, transferable to the recombinants. No such mutation was detected.

Among the lesions which are responsible for permanent inhibition of division in irradiated cells, those of the nucleus are the best known and the most extensively studied. Their role seems to be predominant. In higher organisms, tissue damage is essentially the consequence of chromosomal disorders, not compatible with the normal sequence of mitotic events. The high radiosensitivity of the nucleus is clearly demonstrated in irradiation experiments of *Habrobracon* eggs with  $\alpha$ -rays (Rogers and von Borstel, 1957; von Borstel and Rogers, 1958). Mammalian somatic cells are killed by X-rays according to a sigmoidal dose-effect relationship; two events are necessary to destroy the colony forming ability (Puck and Marcus, 1956; Puck *et al.*, 1957).

In haploid yeast, the X-rays survival curves are exponential, but in diploids they are two-hit sigmoidal ones (Latarjet and Ephrussi, 1949). It has been possible to have direct evidence of the existence of two kinds of genetic lesion. The more frequent are recessive lethal mutations responsible for the sigmoidal shape of the diploid survival curves. The others are dominant lethal mutations (Mortimer, 1955).

In these examples, the lethal effect of X-rays may chiefly be attributed to lesions of the nuclear material.

In *Escherichia coli*, the survival curves are, in practically all instances, exponential. This observation does not depend on the stage of the cultures or on the composition of the growth medium. The bacterial cells possess a variable number of nuclear bodies, according

to the strain and the physiological conditions. Since one event is sufficient to kill *E. coli* cells (permanent division inhibition) the process must either involve a singly existing organelle of the cell or induce some dominant lethal damage in one of many similar entities. If the affected site were one of the nuclei, the lesion might be considered some kind of dominant lethal mutation.

The experiments that will be reported in this paper aim to examine the second alternative, e.g. a dominant lethal mutation in the genetic material.

#### PRINCIPLE OF THE EXPERIMENTS

The material and the genetic methods utilized are those described in the monograph by Wollman and Jacob (1960). The properties of sexual recombination in *E. coli* K12 which will be used in this work are the following:

1. The characters are transmitted from the Hfr donor bacteria to the F<sup>-</sup> recipient cells with a frequency directly proportional to their distance from the origin "O" of the chromosome. Their sequence is always the same for a given Hfr strain.

2. No cytoplasmic material has yet been detected to pass from the Hfr to the F<sup>-</sup> bacteria.

3. During conjugation, the parent cells may be separated by shaking the suspension vigorously; the transfer of the markers still remaining in the Hfr is then interrupted.

Let us call  $\alpha$  the probability, per unit of dose, for the induction of a dominant lethal mutation anywhere in any one of the Hfr chromosomes. The equation of the survival curve to X-rays would be

$$\frac{n}{n_0} = e^{-\alpha D} \quad (1)$$

where  $n/n_0$  is the proportion of surviving bacteria to  $D$  rads. Let us assume that the probability for a given chromosome to be involved in a cross does not depend upon whether or not it carries a dominant X-ray induced lethal mutation. If we assume, in addition, that the transfer to the zygote of this hypothetical mutation will, sooner or later, kill all the recombinants, the expected dose-effect relationship for the survival of recombinants derived from a mating between an irradiated Hfr bacterium and a normal F<sup>-</sup> one is

$$\frac{n}{n_0} = e^{-H/N \alpha D} \quad (2)$$

where  $N$  is the number of chromosomes,  $t$  the probability that the postulated dominant lethal should be transmitted to the zygote, and  $l$  the probability that it would be expressed in the recombinants. According to equation (2), the induction of a dominant lethal mutation in the Hfr chromosomes will allow some predictions which may be experimentally tested:

1. The survival curve of the recombinants will be exponential and have a smaller slope than the survival curve of the Hfr.

2. If the cross is interrupted, the factor  $t$  would decrease (lower probability of transfer of the lethal mutation) and the slope of the recombinants survival curve would be the more diminished, the earlier the interruption.

3. Equation (2) may apply to any kind of selected marker, regardless of the absolute frequency of transfer from the Hfr to the  $F^-$  bacteria. It is also valid for the case of non-selected plating; if the irradiation induces, in the Hfr chromosomes, dominant lethal genes, which kill the selected recombinants to which they are transmitted, they will also express themselves in a decrease of the number of the viable  $F^-$  cells when plated, after a cross, on complete agar.

## MATERIALS AND TECHNIQUES

### *Bacterial strains*

These have kindly been supplied by Drs. Jacob and Wollman. They are: *E. coli* K12 HfrH ( $B_1^-$ , Ss); *E. coli* C600B34-5 ( $T^-$ ,  $L^-$ ,  $B^-$ ,  $Gal^-$ ,  $Sr$ ); and *E. coli* PA309 ( $T^-$ ,  $L^-$ ,  $Try^-$ ,  $H^-$ ,  $Ar^-$ ,  $B^-$ ,  $Lac^-$ ,  $Gal^-$ ,  $Man^-$ ,  $Xyl^-$ ,  $Mal^-$ ,  $T^+$ ,  $Sr$ )

The selected marker in the crosses utilized in this work are  $T^+ L^+ Sr$ . In our experimental conditions, the bacterial cells have two to three Feulgen-positive bodies, the more frequent number being two.

### *Media*

The growth medium is 0.3 per cent Difco Nutrient Broth, 0.5 per cent Bacto Tryptone and 0.5 per cent NaCl. The synthetic medium utilized has been described by Vogel and Bonner. For solid medium Difco Bacto Agar is added to these media at a concentration of 1.5 per cent.

The bacteria are plated using the soft agar (0.8 per cent) technique. Vitamine  $B_1$ , and streptomycin are added in the synthetic soft agar tubes before plating.

## EXPERIMENTAL PROCEDURE

The bacteria are grown in broth up to  $2 \cdot 10^8$  cells/ml and irradiated

in the same medium. 0.1 ml of the Hfr strain is then mixed with 0.9 ml of the F<sup>-</sup> strain, and the mixture slowly agitated at 37°C. Unless otherwise specified, the bacteria are allowed to stand for 1 hr before dilution and plating.

The plates are incubated at 37°C for 48 hr before counting. Irradiation is performed with a Machlett tube AEC 50 with a tungsten target operating at 40 kV. The dose is estimated by comparison with the same lethal effect on the strains observed with a <sup>60</sup>Co source.

## RESULTS

### 1. *HfrH* survival curve and T<sup>+</sup> L<sup>+</sup> Sr recombinants

In our experimental conditions the frequency of T<sup>+</sup> L<sup>+</sup> Sr recombinants formed by the unirradiated controls varies from 20 to nearly 50 per cent.

The survival curves of HfrH and of T<sup>+</sup> L<sup>+</sup> Sr recombinants are exponential; the slope of the latter is less than the slope of the Hfr and depends on the F<sup>-</sup> strain (Fig. 1). Yet the same damage to the HfrH may be quantitatively expressed differently according to the recipient

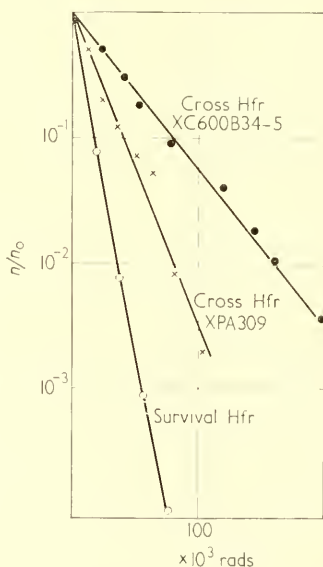


Fig. 1.—*E. coli* HfrH and *E. coli* C600B34-5 are grown in broth up to  $2 \cdot 10^8$  bacteria/ml. The Hfr strain is exposed to increasing doses of X-rays and mated to the F<sup>-</sup> strain: 0.1 ml of the former is added to 0.9 ml of the latter. The suspensions are agitated slowly for 1 hr and then plated on synthetic agar medium, supplemented with all the required growth factors except T and L. The survival curve of Hfr is determined on agar solidified complete medium: the slope is the same if synthetic medium is used.

strain. On the other hand, the difference between the slopes of the two curves may vary slightly from one experiment to another.

## 2. Survival curves of $T^+$ Sr, $L^+$ Sr and $T^+$ $L^+$ Sr recombinants

If the decrease of the frequency of the recombinants with increasing doses of X-rays was related to inactivation of the selected marker, as for instance, mutation from prototrophy to auxotrophy for the controlled substance, the slope of the  $T^+$   $L^+$  Sr recombinant survival curve would be the sum of the slopes of the  $T^+$  Sr and of the  $L^+$  Sr survival curves, assuming that the inactivation of the T and of the L markers are independent events. Figure 2 shows that this is not the case. The

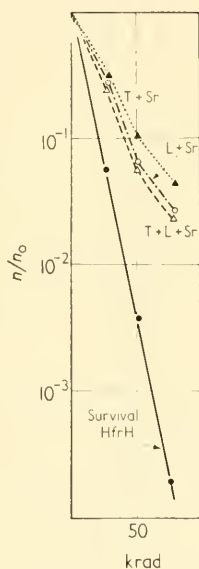


Fig. 2.—The same conditions as for Fig. 1. The crosses are plated on synthetic agar without T and L or with one of each amino acid in order to select for one marker only, or for both.

slopes of the three survival curves are very close: hence the transfer of the two T L markers to the recombinants and its inactivation depend on closely-linked events. This may be demonstrated directly by the genetic analysis of the surviving  $T^+$  Sr and  $L^+$  Sr recombinants. Nearly 100 per cent of them are found to carry the non-selected marker respectively  $L^+$  and  $T^+$ . This result indicates that the loss of the recombinants from an X-rayed Hfr is linked to some damage to the chromosome, apart from the selected markers themselves.



Then, the X-ray lesion of the Hfr expressed in the recombinants must concern the transfer process, being either a damage to the transmission of the genes from the Hfr to the recombinants or a lethal mutation which might kill the zygote. The latter possibility may be tested by interruption experiments.

### 3. *Effect of interruption of conjugation on survival of $T^+ L^+ Sr$ recombinants*

In Fig. 3 are drawn the survival curves of the  $T^+ L^+ Sr$  recombinants for several interruption times. No modification in the slope is observed.

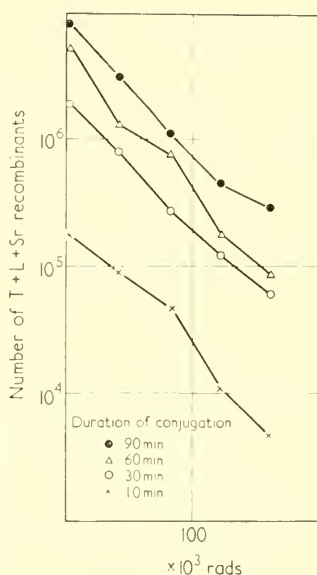


Fig. 3.—The experimental conditions are the same as in Fig. 1. But the matings are allowed to stand for a different length of time and then vigorously shaken and plated on selective synthetic agar.

This eliminates the possibility that a dominant lethal, induced on the chromosome, was transmitted to the zygote at the same time as the  $T^+ L^+$  markers. There remains, however, the alternative that such a hypothetical lethal mutation might exist only on the chromosome segment between the TL loci and the origin "O" and, consequently, will not appear with interruption experiment using these selected markers. The following experiment will answer to this question.

4. *Cross between irradiated Hfr and normal F<sup>-</sup>, plated on non-selective medium*

If a lethal mutation was induced on the Hfr chromosome near the origin "O" it would express itself, in the cross, by the killing of the F<sup>-</sup> bacteria plated on broth medium. The experiment which has been performed consisted of mating Hfr and F<sup>-</sup> cells in the proportion of 10 to 1, so that the non-conjugated F<sup>-</sup> would be very rare. The dose given to the Hfr was such that the probability was low for a mating between an F<sup>-</sup> and an Hfr with an unharmed Hfr chromosome. As the frequency of recombination for the selected markers in this experiment was 30 per cent one would expect at least a 30 per cent decrease in the number of the colonies formed by the F<sup>-</sup> crossed with the irradiated Hfr as compared to the control. No detectable loss in the number of colonies has been observed.

5. *Cross between irradiated F<sup>-</sup> bacteria and normal or irradiated Hfr*

If F<sup>-</sup> bacteria are irradiated and crossed with a non-irradiated Hfr it would be expected that the survival curve of the recombinants and of the F<sup>-</sup> would coincide, on the basis of the hypothesis of a dominant lethal being induced in the F<sup>-</sup>. Many experiments have been done to examine this point. Although for unknown reasons the results lack reproducibility, some restoration of the irradiated F<sup>-</sup> by the Hfr is always observed.

If the cross is made between two irradiated bacteria, Hfr and F<sup>-</sup>, the survival of the recombinants is what might be expected on the assumption that the radiation damages to the Hfr and to the F<sup>-</sup> would act independently in the zygote to reduce the number of the recombinants. This result eliminates the possibility that the exponential inactivation curve for X-rayed cells is the expression of the interaction between a radiation chromosomal event with a radiation cytoplasmic one.

#### DISCUSSION

The experiments reported here have not provided any evidence for the induction of some kind of lethal dominant mutation on the chromosomal material of the Hfr strain. With selected markers, they have shown that a rescue by the F<sup>-</sup> cells may be exerted on the characters of the irradiated male. It seems that the X-ray damage would be, not on the genetic information itself, but mainly on the transfer mechanism of the selected markers to the recombinants. <sup>32</sup>P decay experiments have yielded the same conclusion (Fuerst *et al.*, 1956). Since there exist

differences in the efficiency of rescuing the same selected markers of the Hfr by mating with two different F<sup>-</sup> strains, the slope of the survival curve is of little help, if any, for giving quantitative information on the size of the inactivated target, as has been done in a recent work (Wilson, 1960).

Since no dominant lethals have been found by means of recombination methods two alternative hypotheses may be considered to explain the exponential dose lethal effect relationship in *E. coli*.

1. The lethal effect is not linked to a nuclear damage. Consequently, the inactivated structure should be unique or, if there were many, the alteration of one of them would lead to a dominant lethal sequence of events for which we have no model to propose.

2. The lethal effect is linked to some action in the nuclear material, but is not transmissible to the zygote. Such effects are known. For instance, induction of phage production by lysogenic bacteria is a dominant lethal effect. The cells are killed in relation to phage synthesis and according to a one-hit dose-effect relationship (Marcovich, 1956). On the other hand, induced phage is not transmissible to the recombinants. But this model cannot apply without modification to non-lysogenic or non-inducible lysogenic bacteria. Yet, a starved lysogenic strain loses its aptitude for induction either by u.v. light (Jacob, 1952) or by X-rays (Marcovich, 1957), and still the survival curves are exponential. On the other hand, starvation does not modify the shape of non-lysogenic or non-inducible lysogenic strains and does not affect their intrinsic radiosensitivity. A working hypothesis to explain these results is that the lethal event in *E. coli* and the very early step in lysogenic induction, are processes of the same nature, if not identical. Experiments are now being done to test the implications of this assumption.

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## DISCUSSION

ZHUKOV-VEREZHNICOV: It seems to me that additional proofs are needed that you worked with multinucleate strains of the *Escherichia coli* culture.

MARCOVICH: First, the method of staining according to Feulgen has shown the presence of two or three nuclear bodies. Secondly, if you are working with  $^{32}\text{P}$ -labelled bacteria and allow transmutation to occur, then survival curves to radioactive decay will correspond to multiple-type inactivation, which correlate with the number of bodies stained positively by Feulgen. Finally, if you make crosses, as I have already said, between "male" and "female" strains, and obtain recombination and then remove the "male", you would see that this cell remains normal, because it transmits to the zygote only a part of its genetic material, whereas an identical part of this material remained in it.

ZHUKOV-VEREZHNICOV: What is the largest number of nuclei, which you could distinguish morphologically in one cell in the *Escherichia coli* strain?

MARCOVICH: Up to four in one cell which is preparing to divide. Speaking generally three nuclei may also be encountered, but the number most often met with is two. Experiments aimed at determining the exact shape of the survival curve are such that two nuclei are found with absolute certainty.

ALICHANIAN: I listened to your very interesting and original report with great pleasure. I am interested in a question bearing on the technique of your work. When you were preparing suspensions for inoculation and obtaining recombinants did you take into account the relative proportions of the components, i.e. the parent "male" and "female" cells?

MARCOVICH: Yes, we did. The best method is to introduce one "male" per ten "females", in order to be quite sure that conjugation would occur for all the "males".

ALICHANIAN: Did you use in your work  $\text{F}^{\pm}$  and  $\text{F}^{-}$  or  $\text{Hfr}$  - forms?

MARCOVICH: It may be carried out with  $\text{Hfr}$  forms.

ALICHANIAN: I want once again to emphasize the fact that your work makes it possible to evaluate similar curves on an entirely new basis. This work allows us to decipher dominant and lethal mutations and to determine whether there is death of whole nuclei or some cell damage in general.

GRAY: When crossing occurs there is probably some locus in the chromosome which is first transferred from male to female, and if this is so, then some of the mutations arising during irradiation, would escape analysis.

MARCOVICH: Yes, this is so. If you calculate the target area for this locus, it would be very small. About 1:200 of all the combinations escape, and this is not much.

GRAY: It seems to me that it is important to know what becomes of these chromosomes, because damage may be distributed unequally throughout the chromosome's length.

MARCOVICH: X-rays did not inactivate in these experiments any chromosome locus as such. It was possible only to study the probability of the manifestation of a given locus in the recombinant. Loci for threonine and leucine are found very

close to each other and during the chromosome's injection introduce themselves together. You irradiate and pick out those recombinants which contain both these loci, and obtain that curve which I have drawn.

HERČIK: If you try to take only one of the loci, you would obtain approximately the same curve. It does not mean that the locus itself is damaged but that the probability of its reappearance in the recombinant is decreasing. Does it mean that there is a break in the chromosome?

MARCOVICH: It is possible.

ZHUKOV-VEREZHNICOV: What is the minimum radiation dose to which your system can react with sufficient statistical reliability?

MARCOVICH: The lowest dose accepted in these experiments was 5,000 rad. This material is not suitable for studying smaller doses.

ASTAUROV: Dominant lethals at least in the highest organisms, constitute a complex group of genetic changes. The greater part of them consist of chromosome lesions belonging to the type of deficiencies or breaks. Could it not be that chromosomes with such great lesions would be unable to penetrate at all, and that such disturbances should be left out in this process?

MARCOVICH: It is possible that such breaks may indeed partially explain the death of recombinants. But it seems to me that they could not explain the survival curve. Most likely in bacteria the loss of chromosome material does not lead to the cell's death. In "male" bacteria the loss of chromosome material does not harm them since if crossing occurs the "males" lose part of their nucleus, but do not die.

ARDASHNICOV: Please explain the correction factor.

MARCOVICH:  $\alpha$  is the sensitivity of the whole cell. If  $N$  is the chromosome number, and by accident only one chromosome would be isolated, then the expression of this damage would be  $\alpha/N$ .

ARDASHNICOV: Why do you connect the exponential curve with the presence of dominant lethals? Other factors may account for it, for example, damage to any other unique structure, indispensable for the life of the given organism.

MARCOVICH: I quite agree with you. The only thing I have said is that there are two alternatives, one of them testing the chromosome damage. But it seems to me that my results show that it is not the cause and the damage should be sought elsewhere.



# DAMAGE TO THE REPRODUCTIVE CAPACITY OF HUMAN CELLS IN TISSUE CULTURE BY IONIZING RADIATIONS OF DIFFERENT LINEAR ENERGY TRANSFER

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## SUMMARY

Kidney cells of human origin, cultured by the technique developed by Puck *et al.*, (1956) were irradiated with  $\alpha$ ,  $\beta$ , 200 kV X- and 20 kV X-radiation. The cells were cultured in special dishes with a Melinex bottom  $6\mu$  thick, which permitted irradiation from outside with alpha particles from  $^{210}\text{Po}$  and beta particles from  $^{90}\text{Y}$ . The number of cells, which after irradiation had retained the capacity for clone formation, was counted. The survival curve was found to be exponential in the case of  $\alpha$ -irradiation, whereas with other radiations a more complicated curve was obtained, which cannot be interpreted by a two hit mechanism.

The RBE of  $\alpha$ -radiation was found to range from 2.5 at high doses to 6.0 at low doses. From the experiments with  $\alpha$ -radiation the sensitive area of these cells for the inhibition of clone formation was calculated to be  $40\mu^2$ , which was found to be approximately equal to the cross-sectional area of the nuclei. It may be inferred that clone formation is inhibited if one  $\alpha$ -particle passes somewhere through the nucleus.

Experiments with fractionated doses showed that partial repair takes place after X- and  $\beta$ -irradiation, but no recovery could be detected after  $\alpha$ -irradiation.

Furthermore cells were irradiated with various doses of  $\alpha$ -radiation followed by X-irradiation and conversely. In these experiments no departure from additivity was observed i.e. X-radiation acts on cells surviving after  $\alpha$ -irradiation as if they were not irradiated at all. This contrasts with the effect of a second dose of X-radiation, which depends on the amount of X-radiation the cells have received before i.e. in this case there is some cumulative effect.

Finally experiments are discussed on the sensitivity of these cells to  $\alpha$ - and X-radiation when in equilibrium with different mixtures of nitrogen and oxygen. The absence of oxygen protects the cells in X-irradiation experiments but a very small decrease in sensitivity is found with  $\alpha$ -radiation.

## INTRODUCTION

The development by Puck *et al.* (1956) of a plating technique for single mammalian cells, whereby each cell grows into a separate clone of macroscopic size has stimulated a number of investigations on processes which inhibit this unlimited proliferation. The first experiments

concerning the effects of ionizing radiations on this system were reported by Puek (1959). Using 150 kV X-rays he showed the capacity for clone formation of HeLa cells to be very sensitive to this type of ionizing radiation, with an LD<sub>37</sub> of the order of 100 roentgens. Hood *et al.* (1959) obtained a similar survival curve with 25 kV X-radiation.

Elkind and Sutton (1959) studied the survival of cells derived from ovarian tissue and lung tissue of a Chinese hamster, after fractionated doses of 55 kV X-radiation. They found a definite repair of accumulated damage by surviving cells before their first post-irradiation division.

In the experiments to be described in this paper the effect of ionizing radiations of different linear energy transfer (LET) was studied on the capacity for clone formation by kidney cells of human origin. The survival curve was found to be exponential in the case of  $\alpha$ -radiation whereas for radiations of low LET a more complicated curve was obtained.

Experiments with fractionated doses showed that partial repair takes place after X- and  $\beta$ -irradiation but no recovery could be detected after  $\alpha$ -irradiation. Furthermore the effect was studied of various doses of  $\alpha$ -radiation followed by X-irradiation and conversely. Finally it was shown that the effect of oxygen on the radiosensitivity of these cells is much smaller for  $\alpha$ -radiation than for X-radiation.

#### MATERIALS AND METHODS

In all experiments, kidney cells of human origin (van der Veen *et al.*, 1958) were used, subcultured many times in glass bottles. The culture medium consisted of Hank's solution with 0.5 per cent lactalbumin hydrolysate and 5 per cent calf serum to which 100 IU penicillin and 0.1 mg streptomycin per ml were added. The incubator, at 37°C, was continuously flushed with air, saturated with water vapour and containing 3 per cent CO<sub>2</sub> to maintain the pH of the culture medium at 7.4.

The cells used in irradiation experiments were obtained from four days old flask cultures in the proliferation phase. They were detached from the glass and dispersed by gentle trypsinization (Puek *et al.*, 1956). The cell suspension obtained was counted in a haemocytometer. Microscopic inspection showed that not more than a few per cent of the cells were present in groups of two or three cells.

The cells were plated on culture dishes, "conditioned" by  $1.5 \times 10^5$  "feeder cells" in 3 ml medium which were made incapable of multiplication by a dose of 4,000 rad of X-radiation (Puek *et al.*, 1956). After

about four hours of incubation at 37°C more than 99 per cent of the cells adhered sufficiently to the bottom of the culture dishes for the experiments to be carried out. During the irradiation experiments the cells were maintained at room temperature, 18 to 22°C. The cultures were then placed in the incubator and the cells allowed to grow and multiply for 14 days at 37°C. Medium was replaced once every five days. After 14 days the cells were stained *in situ* and the number of clones of more than fifty cells was counted. The number of clones has been taken to represent the number of surviving cells. In each experiment the mean value was taken of at least three culture dishes each of which had received the same dose. Plating efficiencies of unirradiated cells ranged from 50 to 100 per cent, with an average of about 80 per cent. The fraction of cells surviving irradiation was calculated as a percentage of the unirradiated controls in the same experiment.

Irradiations were carried out with  $\alpha$ -particles from  $^{210}\text{Po}$  (LET  $\approx 170 \text{ keV}/\mu$ ), 20 kV X-radiation, unfiltered except for a layer of culture medium 1 mm thick, (HVL 0.05 mm Al, LET  $\approx 6 \text{ keV}/\mu$ ), 200 kV X-radiation filtered by 1.5 mm Cu (HVL 1.9 mm Cu, LET  $\approx 2.5 \text{ keV}/\mu$ ) and  $\beta$ -radiation from  $^{90}\text{Y}$  (LET  $\approx 0.3 \text{ keV}/\mu$ ). Because of the low penetrating power of  $\alpha$ -particles from  $^{210}\text{Po}$ , which have an energy of 5.3 MeV and a range in water of  $37\mu$ , the cells were cultured in special dishes with a "Melinex"† bottom about  $6\mu$  thick. This thin melinex permitted irradiation of the cells from outside the culture dishes a few hours after plating, when the majority of the cells adhered to the bottom. These Melinex dishes were used in all experiments and were found to support reliably the growth of each surviving cell into a macroscopic clone, even after irradiation which has been reported to give rise to toxicity in plastic surfaces (Morkovin and Feldman, 1959).

Dose measurements were carried out with various dosimeters. The dose of  $\alpha$ -radiation delivered to the cells was determined by counting the number of particles passing per minute through 1 sq. mm, by measurement of the total current in a large ionization chamber and with an extrapolation chamber. The dose of  $\beta$ -radiation was measured with the extrapolation chamber. The dose of 200 kV X-radiation was measured with a Baldwin "Substandard" ionization chamber and with the extrapolation chamber. The dose of 20 kV X-radiation was measured with a Philips ionization chamber type 37483/01, suited for HVL 0.02–1.5 mm Al.

Details of the irradiation techniques and dosimetry are given elsewhere (Barendsen and Beusker, 1960).

† Polyethylene terephthalate, Imperial Chemical Industries Limited, Herts., England.

## RESULTS

*Survival curves*

In Fig. 1 the fraction of cells which after irradiation have retained the capacity for clone formation is plotted as a function of dose. Doses of  $\alpha$ -,  $\beta$ - and X-radiation are given in rads. The main features of the

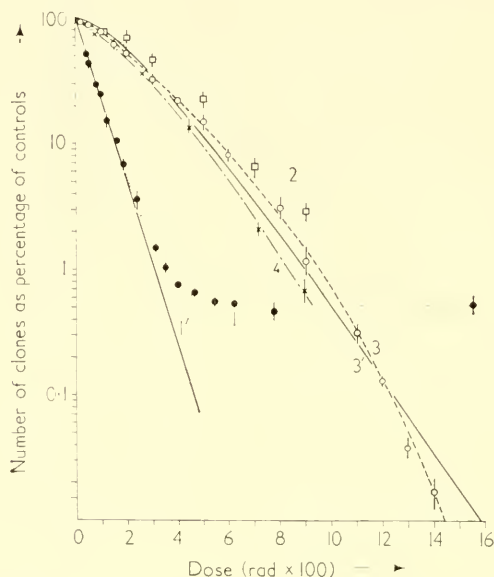


Fig. 1.—Effects of  $\alpha$ -,  $\beta$ - and X-radiation on the capacity for clone formation.

1. Curve obtained with  $\alpha$ -radiation.
- 1', Curve 1 corrected for cells, not adhering to the bottom of the dishes (see text).
2. Curve obtained with  $\beta$ -radiation. RBE  $\approx 0.85$ .
3. Curve obtained with 200 kV X-radiation.
- 3', Theoretical curve  $n/n_0 = e^{-D/135} (1 + D/135)$ . (see text).
4. Curve obtained with 20 kV X-radiation. RBE  $\approx 1.15$ .

curves are the exponentially decreasing survival for  $\beta$ -radiation and the less simple shape obtained with X- and  $\beta$ -radiation.

At doses of  $\alpha$ -radiation of more than 150 rad the survival curve deviates from the exponential. This may be explained by the assumption that a small percentage of the cells, about 0.5 per cent, has not attached properly to the Melinex bottom of the culture dish. As the range of  $\alpha$ -particles after penetrating the Melinex bottom is only about  $20\mu$ , cells which are still in suspension at the time of irradiation will not be irradiated at all. When a constant fraction of 0.5 per cent is subtracted from the surviving fraction at all dosages, an exponential

survival curve is obtained up to at least 400 rad (Fig. 1, curve 1'). The mean lethal dose ( $LD_{37}$ ) is found to be 65 rad.

The exponential survival curve may be represented by

$$\frac{n}{n_0} = e^{-SD}$$

in which  $n_0$  is the number of cells plated,  $n$  is the number of cells surviving a dose of  $\alpha$ -radiation  $D$  and  $S$  is a constant, which may be interpreted as a "sensitive area" if  $D$  is expressed in  $\alpha$ -particles per unit area passing through the cells. Taking an average LET of 170 keV/ $\mu$  for  $\alpha$ -particles of 3.4 MeV, one  $\alpha$ -particle passing per square micron is equivalent to an average dose of 2,720 rad. From the  $LD_{37}$  of 65 rad  $S$  can be calculated to be  $42\mu^2$ , which corresponds to a circular area of  $7.4\mu$  diameter. This very large sensitive area is about equal to the area presented to the  $\alpha$ -radiation by the nuclei of the cells, which, by microscopic examination, were found to range between 6 and  $10\mu$  in diameter. As it is generally accepted that the nucleus of a cell is much more sensitive to ionizing radiation than the cytoplasm, it seems very unlikely that any structure in the cytoplasm can be correlated with this sensitive area of about  $42\mu^2$ . Thus the cross-section of the nucleus must be identical with the sensitive area calculated from the experiments. This suggests that whenever one  $\alpha$ -particle penetrates the nucleus anywhere, the cell is sufficiently damaged to become incapable of unlimited proliferation.

The survival curves obtained with  $\beta$ - and X-radiation are not exponential but the slope of the tangent increases with increasing dose. For the curve obtained with 200 kV X-radiation this slope corresponds at 100 rad to a mean lethal dose ( $LD_{37}$ ) of about 400 rad, at 700 rad to an  $LD_{37}$  of about 180 rad and at 1,300 rad to an  $LD_{37}$  of about 100 rad. It may be noted that as a result of this difference in shape between the survival curves the relative biological efficiency (RBE) of  $\alpha$ -radiation, defined as the ratio of doses of 200 kV X-radiation and  $\alpha$ -radiation which cause the same effect, ranges from 2.5 at 0.017 per cent survival to at least 6 at 80 per cent survival. The survival curve obtained with  $\beta$ -radiation has, up to doses of 900 rad, a shape which is not significantly different from the curve obtained with 200 kV X-radiation (Fig. 1, curve 2). From the results the RBE is calculated to be  $0.85 \pm 0.10$ . The same conclusion may be drawn from curve 4 obtained with 20 kV X-radiation, for which the RBE is calculated to be  $1.15 \pm 0.10$ .

The shape of the 200 kV X-ray survival curve suggests that some sort of accumulation of damage takes place. Puck (1959) assumes for the explanation of a similar curve obtained with 150 kV X-radiation a



two-hit mode of action on the chromosomal material. The theoretical curve expected from this assumption has the form

$$\frac{n}{n_0} = e^{-D/A} (1 + D/A)$$

where  $n/n_0$  is the relative survival after a dose  $D$  and  $A$  is a constant. In Fig. 1, curve 3' is an example of this type of curve in which  $A$  is chosen as 135 rad in order to obtain the best fit with the experimental points. The accuracy of our experiments is sufficient to conclude that the assumption of a two-hit mode of action as proposed by Puek is too simple. It may be noted in passing that from the assumption of a two-hit mode of action it may be inferred that the slope of the survival curve at low doses approaches zero. This would imply that for this system the RBE of  $\alpha$ -radiation at low doses would approach infinity. However, our results at low doses show an initial slope corresponding to an LD<sub>37</sub> of about 450 rad.

An hypothesis by which our survival curves may be explained starts from the consideration that the single-hit survival curve obtained with  $\alpha$ -radiation suggests that deposition of a sufficiently large amount of energy in a small volume anywhere in a relatively large part of the nucleus inhibits clone formation.

Consideration of the spatial distribution of the energy deposition by X-radiation makes plausible that at least a small part of the damage caused by ionizing radiations of low average LET will be due to the same type of locally concentrated energy deposition i.e. part of the X-ray damage may be considered to be caused by a single-event type of action. The greater part of the energy, which is deposited in less concentrated form, may be assumed not to result in inhibition of clone formation. The damage caused by this part of the energy deposited might be repairable (see next section). At higher doses of X-radiation two or more amounts of energy deposited in small volumes, each by itself too small to inhibit clone formation, may be found sufficiently close together to cause this effect. Thus with increasing dose a greater part of the energy deposited is effective in causing the damage measured. In this way the shape of the X-ray survival curve with increasing slope at higher doses may be explained as a combined result of one-, two- and multi-event types of action.

### *Effects of fractionated doses*

A number of experiments was carried out in which doses of X-radiation up to 900 rad and doses of  $\alpha$ -radiation up to 200 rad were fractionated in such a way that the second half of the total dose was

administered after intervals of 2, 4 and 12 hr. In the interval the cells were maintained at 37°C.

With  $\alpha$ -radiation no statistically significant differences were observed between the effects of single doses of respectively 50, 100 and 200 rad and of two doses of respectively 25, 50 and 100 rad administered at intervals of 2, 4 and 12 hr. At doses up to 500 rad of 200 kV X-radiation and intervals of 2 and 4 hr the effect of fractionation was very small. At higher doses and with longer intervals the effect is significant however. For example the surviving fraction after a dose of 900 rad was found to be  $1.3 \pm 0.2$  per cent, whereas after two times 450 rad, 12 hr apart,  $4.0 \pm 0.05$  per cent of the cells had retained the capacity for clone formation (see Fig. 2). Thus more cells survive after fractionated

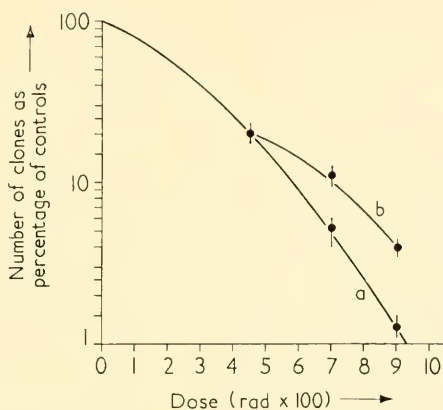


Fig. 2.—Effect of fractionation of doses of 200 kV X-radiation on the capacity for clone formation.

Curve 1, Surviving fraction after irradiation 4 hr after plating.

Curve 2, Surviving fraction after 450 rad at 4 hr after plating + 250 rad and 450 rad respectively, administered 12 hr after the first dose.

doses i.e. part of the damage is repaired in the interval. The higher survival was not due to cell multiplication in the interval, as cells irradiated with 900 rad of 200 kV X-radiation 16 hr after plating showed a surviving fraction of  $1.5 \pm 0.1$  per cent. These results are in agreement with the work of Elkind and Sutton (1959) except that in our system repair appears to start somewhat later.

#### *Effects of combination of doses of $\alpha$ - and 200 kV X-radiation*

In Fig. 3 survival curves are given from experiments in which the same cells were irradiated with X- and  $\alpha$ -radiation. No statistically

significant differences in effect were observed if the order in which  $\alpha$ - and X-irradiations were given was reversed. It will be clear from curves 1 and 2 that if a certain dose of  $\alpha$ -radiation is given first, the curve

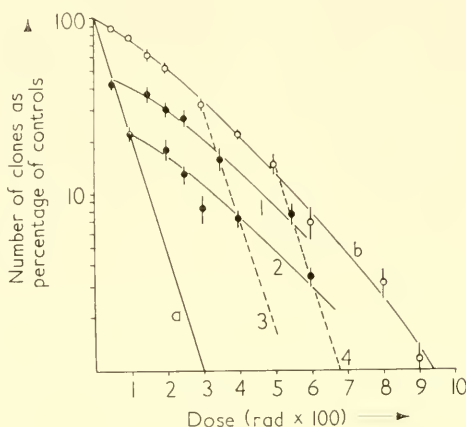


Fig. 3.—Effects of combined  $\alpha$ - and 200 kV X-radiation on the capacity for clone formation.

a and b, Effects of  $\alpha$ - and 200 kV X-radiation respectively.

Curve 1, Effects of 50 rad  $\alpha$ -radiation + O; 100; 150; 200; 300 and 500 rad of 200 kV X-radiation.

Curve 2, Effects of 100 rad  $\alpha$ -radiation + O; 100; 150; 200; 300 and 500 rad of 200 kV X-radiation.

Curve 3, Effects of 300 rad 200 kV X-radiation + O; 50 and 100 rad  $\alpha$ -radiation.

Curve 4, Effects of 500 rad 200 kV X-radiation + O; 50 and 100 rad  $\alpha$ -radiation.

obtained from X-irradiation of the surviving cells has the same shape as if no preceding  $\alpha$ -irradiation had occurred, i.e. X-radiation acts on cells surviving  $\alpha$ -irradiation as if they were not irradiated at all. This contrasts with the effect of two doses of X-radiation where the damage, at least in part, is cumulative. The dotted lines 3 and 4 parallel to the survival curve a obtained with  $\alpha$ -radiation indicate that, within limits of error, cells surviving after X-irradiation have not become more sensitive to  $\alpha$ -radiation i.e. in this respect there is no cumulative effect. This supports the conclusions given in the first section.

#### *Effects of different oxygen concentrations on radiosensitivity*

A reduction in the sensitivity to ionizing radiation resulting from anoxia has been reported for many systems (Bora, 1958; Gray *et al.*, 1958; Neary *et al.*, 1959). In order to measure the effect of different oxygen concentrations on the capacity for clone formation, the medium was removed from the culture dishes after the cells had attached to the

Melinex bottom. Gas mixtures containing different amounts of  $O_2$  and  $N_2$  saturated with water vapour were then passed over the cells for various lengths of time. No differences in reduction of the sensitivity to X-radiation were observed when the gas mixtures were passed over the cells for 5, 10, 15 and 25 min respectively. It was concluded that a time of 5 min was sufficient for the cells to reach equilibrium with the gas phase. In the experiments summarized in Table I gas was passed

TABLE 1.—*Relative numbers of cells as percentage of unirradiated controls, which have retained the capacity for clone formation after irradiation in gas mixtures of various proportions of oxygen and nitrogen.*

X-radiation medium removed		Gas mixture (10 min)		600 rad	1,000 rad
—		no gas		8.0 ± 0.5	0.6 ± 0.1
+		passed over		9.3 ± 1.5	0.7 ± 0.4
+		air		13.3 ± 1.7	1.4 ± 0.6
+		100 % $O_2$ +	0 % $N_2$	12.4 ± 1.7	0.2 ± 0.2
+		50 % $O_2$ +	50 % $N_2$	12.8 ± 1.8	0.7 ± 0.4
+		20 % $O_2$ +	80 % $N_2$	13.5 ± 1.8	1.4 ± 0.6
+		10 % $O_2$ +	90 % $N_2$	18.8 ± 2.6	0.9 ± 0.5
+		5 % $O_2$ +	95 % $N_2$	20.4 ± 2.2	2.8 ± 0.8
+		2.8 % $O_2$ +	97.2 % $N_2$	22.6 ± 2.3	4.0 ± 1.0
+		1.5 % $O_2$ +	98.5 % $N_2$	24.4 ± 2.4	8.4 ± 1.4
+		0.9 % $O_2$ +	99.1 % $N_2$	32.8 ± 2.8	11.8 ± 1.7
+		0.3 % $O_2$ +	99.7 % $N_2$	43.9 ± 3.1	20.8 ± 2.4
+		0 % $O_2$ +	100 % $N_2$	65.8 ± 5.6	27.6 ± 3.5

Alpha radiation medium removed		Gas mixture (10 min)		100 rad	150 rad	200 rad
—		no gas		22.0 ± 1.5	10.6 ± 0.8	5.0 ± 0.5
+		passed over		24.7 ± 2.3	12.4 ± 1.7	3.5 ± 0.9
+		air		28.7 ± 2.5	13.9 ± 1.7	7.3 ± 1.2
+		99.7 % $N_2$ + 0.3 % $O_2$		33.1 ± 2.7	17.9 ± 1.9	8.6 ± 1.5

over the cells for 10 min before the irradiation was started. In this table surviving fractions are given for oxygen concentrations in the mixture of 100, 50, 20, 10, 5, 2.8, 1.5, 0.9, 0.3 and 0 per cent. The nitrogen from the cylinder used was found to contain 0.3 per cent  $O_2$ . This oxygen was removed with a "BTS-katalysator"†. It may be concluded that with oxygen concentrations between 10 and 100 per cent no statistically significant differences in radiosensitivity are observed. With gas mixtures which contain less than 5 per cent  $O_2$  the radiosensitivity of the

† Badische Anilin und Soda Fabrik.

capacity for clone formation decreases with decreasing oxygen concentration. It may be noted that there is a slight difference in radiosensitivity between cells over which no gas mixture is passed at all and cells which are in equilibrium with air. An explanation of this effect cannot as yet be given (see Fig. 4).

Comparison of the curves given in Fig. 4 shows that with an oxygen

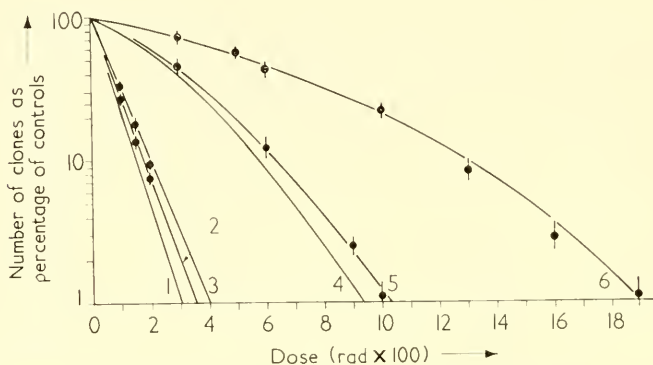


Fig. 4.—Effects of  $\alpha$ - and 200 kV X-radiation on cells in equilibrium with air and nitrogen respectively.

- 1, Curve obtained for  $\alpha$ -radiation under normal conditions with medium not removed (see Fig. 1).
- 2, Curve obtained for  $\alpha$ -radiation with cells in equilibrium with air (medium removed).
- 3, Curve obtained for  $\alpha$ -radiation with cells in equilibrium with 99.7%  $N_2 + 0.3\%$   $O_2$  (medium removed).
- 4, Curve obtained for 200 kV X-radiation under normal conditions with medium not removed (see Fig. 1).
- 5, Curve obtained for 200 kV X-radiation with cells in equilibrium with air (medium removed).
- 6, Curve obtained for 200 kV X-radiation with cells in equilibrium with 99.7%  $N_2 + 0.3\%$   $O_2$  (medium removed).

concentration of 0.3 per cent as used in most of the experiments, a reduction of the sensitivity was obtained by a factor of about 2 as compared with the sensitivity observed with oxygen concentrations between 10 and 100 per cent i.e. twice as high a dose is needed to produce the same effect. With purified nitrogen a reduction of the sensitivity by a factor of 2.6 was found.

Experiments carried out with  $\alpha$ -irradiation of cells in equilibrium with 99.7 per cent  $N_2 + 0.3$  per cent  $O_2$  showed that with this radiation of high LET only a very slight decrease in radiosensitivity by a factor of about  $1.10 \pm 0.05$  can be achieved as compared with aerated cells (see Table I and Fig. 4). This result is in agreement with that found



with other systems namely that the oxygen effect is smaller for radiations of high LET than for radiations of low LET (Bora, 1958; Gray *et al.*, 1958; Neary *et al.*, 1959).

In Fig. 5 the sensitivity of these cells to 200 kV X-radiation is given as a function of the oxygen concentration, whereby the sensitivity of cells in equilibrium with pure nitrogen is taken as unity. The

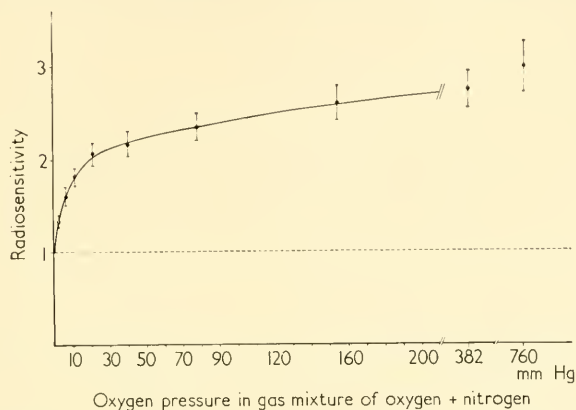


FIG. 5.—Sensitivity to 200 kV X-radiation of human cells in equilibrium with gas mixtures which contain different percentages of  $O_2$  and  $N_2$ . Sensitivity of cells in equilibrium with pure nitrogen is taken as unity.

shape of this curve is very similar to curves obtained with other systems (Gray *et al.*, 1958; Howard-Flanders, 1958). It can be represented approximately by

$$\frac{S}{S_n} = \frac{K + m[O_2]}{(K + [O_2])}$$

where  $S$  is the radiosensitivity ( $1/D_{37}$ ) of cells in equilibrium with oxygen concentration  $[O_2]$ ,  $S_n$  is the radiosensitivity in nitrogen and  $m$  and  $K$  are constants. The value found from our curve for  $K$  is about  $7 \mu\text{moles/l.}$

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#### DISCUSSION

HERČIK: What is the thickness of the cells. I mean cells lying on the dish bottom in experiments with  $\alpha$ -particles?

BARENDSEN: The residual range of the  $\alpha$ -particles is about  $25\mu$ . The thickness in vertical direction of the cells attached to the bottom because of flattening, ranges from 5 to  $15\mu$  so that each part of the cells is irradiated.

POWERS: How do you explain differences between the survival curves for X-rays and  $\alpha$ -irradiation?

BARENDSEN: As I see it the nucleus is the most sensitive site of the cells and in order to answer your question we have to consider by what mechanism the damage is produced. Alpha particles passing through the nucleus will inevitably damage one or more chromosomes, the integrity of which is indispensable for the reproduction of the cells. X-rays will produce the same damage (due to local deposition of sufficient energy) less efficiently because of the lower LET and other damage (due to less than the required number of ionizations in a small volume) may be partially restored, possibly by the metabolic activity of the cells. A possible mechanism might be that a large amount of energy deposited leads to changes in DNA such that the separation of the two strands of the helix is made impossible. If the amount of energy is not large enough the DNA can still separate into two strands, and by this action the lesion is repaired. At higher doses the probability that two or more electrons pass through the same DNA molecule and together produce sufficient damage to prevent strand separation increases. This produces an increasing efficiency of sparsely ionizing radiation at higher doses and leads to a survival curve of the type observed with X-radiation. Thus the primary lesions are produced at the time of irradiation, but the final damage may be influenced by the metabolic activity of the cell.

PASSYNSKY: Did the result obtained for  $\alpha$ -irradiation depend on the mitotic phase? Have any differences been found between prophase and metaphase?

BARENDSEN: This could not be determined since the cell divisions were not synchronous.

TOBIAS: Why was the presence of 50 cells in the clone chosen as a survival criterion?

BARENDSEN: We tried to take 20 cells, 100 and 200 cells, and found no difference. Every surviving cell will multiply, and from it several thousand cells will arise.

TOBIAS: I am very happy to hear that the number of cells is of no importance. But, in the case of yeast, colonies sometimes die out, even if they contain 50 cells.

BARENDSEN: We did not observe this.

SOŠKA: What was the composition of the medium used for the kidney cells?

BARENDSEN: The medium consists of Hank's solution with 0.5 per cent laetalbumin hydrolysate and 5 per cent calf serum to which 100 IU of penicillin and 0.10 mg of streptomycin per ml were added.

ARDASHNICOV: How did you calculate the dose for  $\alpha$ -irradiation? If you get a one-hit curve why, in this case, are doses for  $\alpha$ -irradiation so much smaller than for X-rays? Maybe in this case you take for a hit an  $\alpha$ -particle, and not the ionization it produces?

BARENDSEN: I used two methods to determine the dose. First of all I counted the number of particles per square mm. Secondly, I also used an ionization chamber placed where the layer of water is situated during the irradiation.

# PHOSPHATE METABOLISM IN THE NUCLEUS

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## SUMMARY

A brief account is given of the effects of X-radiation on the biochemical events in the mitotic cycle leading to the formation of nucleic acids. It is suggested that low doses produce a disorganization of binding sites in the nucleus. Preliminary data on the capacity of the nucleus to bind inorganic and organo-phosphates are provided.

This paper is a brief survey of what may be the salient biochemical features associated with the synthesis of DNA in animal cells and an attempt to see how far the derangement of DNA synthesis by X-radiation can be explained in the light of our present knowledge. We ourselves are convinced of the lack of basic information about the nucleus and for this reason some of our recent findings which we have not yet applied in the radiation field will be reported.

The early work on the inhibition of precursor uptake into DNA is due to Hevesy (1948). Most of these data were obtained at 2 hr after irradiation and have been subjected to the criticism that by this time changes in cell population or cell death have complicated the picture. From our experiments (Ord and Stocken, 1957) with thymus gland, however, it does seem that radiation produces its effect at once and there is no further change until some considerable time afterwards.

A second interesting observation due to Hevesy is the fact that once the extent of inhibition has reached about 50 per cent a considerable increase in the dose is required to produce much further change. We followed up this point and correlated the dose with the inhibition of  $^{32}\text{P}$  uptake into thymus DNA (Ord and Stocken, 1958). It seems clear from these experiments that radiation has at least two separate functions. A similar biphasic response has been found by Lajtha *et al.* (1958) when bone-marrow cultures are irradiated. In order to explain the results it is necessary to seek a sensitive as well as a rather resistant locus.

The first clue to differential sensitivity of cells stemmed from the work of Howard and Pelc (1953) on the radiation sensitivity of the mitotic cycle in bean root tips. These authors showed that the cycle

could be divided into discrete periods which they called  $G_1$ , S,  $G_2$  and D.

To produce the same inhibition of uptake of DNA precursors several times the dose is required if it is given in S than if the same dose is given in  $G_1$ . Similar results have been obtained in regenerating liver by Kelly (1957) who used carbon tetrachloride to destroy liver cells and by Holmes and Mee (1956) who surgically removed two-thirds of the liver. Barbara Holmes also showed that if 450 r was given just before partial hepatectomy there was the same inhibition and mitotic delay as when the dose was delivered at 12 hr after hepatectomy.

So far as mitotic delay is concerned one should perhaps remember that Carlson (1948) has shown that an arrest can be caused by as little as 4 r and Forssberg and Novak (1960) that doses of 0.1 r and less affected the growth of *Phycomyces Blakesleanus*.

We have very little information about the biochemical events taking place during the process of division but we now know quite a lot about the biochemistry of interphase in mammalian cells. During the first part of the cycle there is an early stimulation of RNA and protein synthesis. This has been shown both *in vivo* and *in vitro* at 6 hr after partial hepatectomy. This is followed by the appearance of thymidylate kinase and DNA polymerase (Bollum *et al.*, 1960) and the synthesis of DNA does not start until about 15 to 18 hours post hepatectomy. It is also to be noted that at the same time as the synthesizing enzymes appear there is a reduction in the pyrimidine catabolizing enzymes.

The effect of radiation on these various steps is of some interest. If the irradiation is given before the time at which the enzyme can be detected then its appearance is delayed, but when the enzyme is present the same radiation dose has very little effect. In view of the relationship of RNA with protein and enzyme synthesis it is of interest that Welling and Cohen (1960) have observed a decreased incorporation of  $^{32}\text{P}$  into nuclear RNA of regenerating rat liver if the animal is irradiated within 6 hr of partial hepatectomy. There is also a delay in the disappearance of the catabolizing enzymes. Okada and Hempelmann (1959) have data at 12 hr post-hepatectomy and a more complete time course has been obtained by Stevens in our laboratory (Table I). This delay, of course, only applies in comparatively low doses and when lethal doses are given it is likely that these changes are made irreversible by more fundamental damage, such as an alteration in the template.

Cole and Ellis (1954) have observed changes in spleen nucleoprotein after irradiation *in vivo* and by means of column chromatography we have found changes in thymus DNA (Old and Stocken, 1960). Recently Mrs. Hudnik-Plevnik in our laboratory has also obtained preliminary

results with bacterial DNA after exposure to ultraviolet irradiation and shown that the newly synthesized DNA is not the same as that in the controls.

The consequences of severe doses of radiation are of biochemical interest but it seems unlikely that we shall in the near future be able

TABLE 1. *The effect of 400 r X-rays total body irradiation on the thymine catabolizing enzymes in regenerating rat liver*

Hours post-partial hepatectomy	Control	Irradiated
Non operated	2.5 (4)	—
18	2.4 (4)	—
24	0.8 (4)	2.2 (8)
36	0.6 (2)	—
48	0.55 (2)	1.65 (8)

$\mu$ moles catabolized/mg DNAP/30 min.

to reverse the effects of supra-lethal doses except by modifications of replacement therapy. On the other hand if we knew exactly what was the biochemical lesion caused by the near-lethal doses we might be able to promote recovery without the implantation of extraneous cells.

Our present opinion is that low doses produce a local disorganization of the nucleus which alters the binding sites. Some support for this idea is given by Hagen's (1960) recent work on the extractability of DNA from thymus gland homogenate post radiation, and by Creasey (1960) who found a leakage of  $\text{Na}^+$  and  $\text{K}^+$  from nuclei of rat thymus and spleen at one hour after 1,000 r given *in vivo*. Smaller doses were effective when the nuclei were irradiated *in vitro*.

It is this question of what maintains the nucleus in an organized state which has caused us to concentrate on some of the basic properties of the nucleus.

Allfrey *et al.* (1957a) have extensively studied protein synthesis in isolated nuclei and shown that  $\text{Na}^+$  is an essential cation. They have also discovered nuclear phosphorylation (1957b) and it may be conjectured that this might be the energy source for intra-nuclear synthesis.

We have been concerned with another aspect of phosphorus metabolism in thymus nuclei. When nuclei are prepared in either ionic or sucrose medium both inorganic and organo-phosphate is bound to the nucleus. These phosphates are only released by acid conditions or by such severe mechanical damage that the nuclear structure is no longer maintained.

If a rat is killed and the thymus removed within a few seconds the isolated nuclei contain phosphate predominantly as ATP. If the thymus



is left in the dead rat for a short time the ATP content declines. This of course is not unexpected but it is of interest that the bound inorganic phosphate increases roughly in an inverse ratio. Allfrey *et al.* (1957b) have pointed out that the phosphorylation of mononucleotide is not effected by extranuclear inorganic phosphate and with this we are in agreement.

We have carried out some experiments with thymus nuclei from rats killed at various times post injection of  $^{32}\text{P}$ . The rather surprising finding is that the bound inorganic phosphate of the nuclei has a markedly higher specific activity than any other cell fraction. This

Table II. *Specific activities of acid-soluble phosphates in rat thymus after injecting 50  $\mu\text{C}$   $^{32}\text{P}$  intramuscularly/100g body weight*

Time after injection (min.)	Mean SA plasma $\text{P}_1$	SA DNA $\times 10^3$	Cell sap		Specific activities				
			$\text{P}_1$	$\text{P}_{15}$	Microsomes $\text{P}_1$	Mitochondria $\text{P}_1$	$\text{P}_1(\text{sol})$	Nuclei $\text{P}_1(\text{b})$	$\text{P}_{15}$
3	2000	—	—	—	—	—	—	—	—
5	—	388	79	—	90	145	97	158	—
10	1130	800	185	128	—	—	—	—	—
10	—	785	255	142	—	253	187	377	133
20	—	1580	356	156	—	—	235	548	259
20	—	—	271	—	—	—	—	447	—
30	260	3750	318	269	—	—	265	—	257
30	—	3020	329	177	—	—	195	425	264
60	120	7050	351	206	—	—	—	—	289
60	—	—	—	—	—	—	93	288	187
60	—	7200	—	—	—	—	156	—	191
60	—	5333	206	—	194	318	—	270	—
60	—	5170	212	—	174	304	—	269	—
120	—	—	211	170	—	—	—	218	213
240	—	—	158	118	—	—	—	190	158

Specific activity as counts per min per  $\mu\text{g}$  P (SA)

may be due to differences in accessibility of organo-phosphates in different cell compartments to enzymic hydrolysis.

Data for mitochondria are less easy to obtain since two separate preparations have to be made to obtain nuclei and mitochondria. Experiments *in vitro* showed that the bound  $\text{P}_1$  was exchangeable with added  $\text{P}_1$  and incubation of radioactive nuclei with inert  $\text{P}_1$  or of inactive nuclei with  $^{32}\text{P}_1$  shows the expected changes in specific activity.

We have lately been preoccupied to find a more controlled system for the study of penetration of ions to the nucleus and preliminary experiments indicate that a suspension of isolated thymocytes will prove useful.

As I mentioned earlier these experiments have been undertaken to provide more basic information about the metabolism of the nucleus and we have not yet investigated the consequences of exposure to ionizing radiation.

### ACKNOWLEDGMENT

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### DISCUSSION

KUZIN: You have said that very soon, as early as 10 min following radiation exposure, changes in the DNA are observed when it is studied by chromatographic methods using ECTEOLA cellulose. Are you sure that the DNA was pure? According to the data you have presented, the nitrogen/phosphorus ratio in your DNA preparation is so high that considerable protein contamination may be suspected, and hence, contamination by DNAases.

Is it not possible that this contamination accounts for the rapid depolymerization you have observed?

STOCKEN: It is difficult indeed to determine the purity of the DNA preparations. We tested for protein by the Folin-Ciocalteu test and obtained a negative result which indicates less than 0.5 per cent contamination. It should also be noticed that both samples were prepared simultaneously and by the same procedure. At the same time we cannot say that the DNA preparations used were quite pure and contained no admixtures whatsoever.



# INITIAL STEPS IN RADIATION DAMAGE TO CHROMOSOMES AND MEANS OF PREVENTING THIS EFFECT

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## SUMMARY

Two approaches to the analysis of the initial steps in radiation damage are discussed.

The first is the use of very low radiation levels. Thus the entire effect of small doses of radiation on the grasshopper neuroblast can be reversed by immediate treatment with hypertonic salt solution. X-ray-induced chromosomal aberrations in *Tradescantia* pollen may be modified by pre- and post-treatment with u.v.

The second approach is the investigation of protection against radiation damage. The killing of *A. terreus* cells and mutation are not interdependent. Pre-mutation damage in *Paramecium* can be repaired.

All these studies establish that it is possible to manipulate living cells in regard to survival, mitosis, chromosome breaks and mutation production by radiation.

The identification of basic mechanisms involved in the initial effects of radiation on living cells has been actively investigated in our laboratory in the past few years. The emphasis, fitting into the general programme of the laboratory, has been on cytological and genetic effects. Some of our projects, discussed here, give a picture of what we know about the effects of ionizing radiation on chromosomes and suggest certain types of investigations which are essential.

In an analysis of the initial steps involved in radiation damage, we have used two approaches. One approach has persuaded us to go to lower and lower radiation levels and search for effects which we can determine quantitatively. Referred to here are only the *initial* steps of radiation damage. Of course, when massive doses of radiation are administered many changes in living cells occur. But in this case steps in damage follow each other so rapidly that it is usually not practicable to analyse the initial steps since they are quickly obscured.

The other approach is to investigate the areas where we can protect against radiation damage. Special emphasis has been placed on what can be done to help the cell repair the damage which radiation has initiated before it becomes frozen or starts a chain reaction which

† Operated by Union Carbide Corporation for the United States Atomic Energy Commission.

cannot be interrupted. The fields in which we are most interested—cytological and genetic effects—readily lend themselves to quantitative analysis. This type of damage may have very serious long-term effects and is of greatest importance to the sensitivity of man to radiation damage.

The first problem is the effect of very small quantities of radiation on the rate of mitosis. The immediate effect of less than 30 r is a slowing down of the mitotic rate. I refer to the work of Mary Esther Gaulden of Oak Ridge National Laboratory and J. Gordon Carlson of The University of Tennessee. Since cytological changes produced by radiation can now be observed readily in living cells, a method has been developed to follow the rate of mitosis in the grasshopper neuroblast under constant, carefully controlled conditions. In this case the rate of mitosis is very reproducible and can very well be used as a criterion of radiation damage. The grasshopper neuroblast is suspended in a nutrient medium in a hanging drop slide and observed under constant temperature conditions under the microscope. The rate of damage is determined by observation about every 20 min. For details I refer you to the appropriate publications for I will discuss here only the initial effects of radiation. A small amount of radiation—less than 25 r and even an effect of as little as 1 to 3 r can be observed—results in a slowing down of the rate of mitosis. This slow-down is followed by a period of compensating speed-up after a period of inhibition.

However, we have found that we can reverse the entire effect by treatment with hypertonic salt solution. Immediately after irradiation—less than 60 sec later—we treat the cells with a slightly ( $1.2 \times$  the isotonic salt concentration) hypertonic culture medium. The hypertonic salt solution itself has the effect on normal cells of doubling the rate of mitosis. In cells irradiated with 3 r of X-rays the solution obscures entirely the slowing down effect of the radiation. Irradiation only affects mitotic rate of middle and late prophase cells. Inasmuch as Gaulden (1956) has observed that the grasshopper neuroblast synthesizes deoxyribonucleic acid (DNA) only during the period from middle telophase to very early prophase, it is obvious that the slow-down effect must lie outside the area of nucleic acid synthesis. It appears that the initial effect of radiation in this case is a purely physical and not a chemical one. It should be pointed out that at these low levels of radiation the slowing of the mitotic rate is not influenced by oxygen.

The next project for discussion is the induction of chromosomal aberrations in *Tradescantia* pollen, a synergistic effect between very small amounts of combined X-ray and ultra-violet (See Table I). This is the work of J. S. Kirby-Smith, Benedetto Nicoletti, and M. L. Gwyn



TABLE 1. *Typical results showing the synergistic action of ultra-violet and X-radiation*

	Dose	Cells scored	Isochromatic (%)
<i>Expt. 1-9 E</i>			
X-ray	50 r	250	4.4
u.v.	$0.1 \times 10^6$	300	2
X-ray-u.v.		300	18
<i>Expt. 1-7 E</i>			
X-ray	50 r	200	4.5
u.v.	$0.1 \times 10^6$	115	5
X-ray-u.v.		146	112

(1960). These investigators have observed that it is possible to modify X-ray induced chromosomal aberrations by pre- and post-treatment with monochromatic u.v. radiation. The most extensive observations in this field have been made with X-rays in the dose range 50 to 150 r and ultraviolet exposure between 50,000 and 200,000 ergs/cm<sup>2</sup>. The technique is to irradiate pollen under carefully controlled conditions of humidity and examine chromosomal aberrations at metaphase in the post-meiotic mitosis in pollen tube cultures. The magnitude of the effect depends in a complex manner on the time intervals between exposure to the different radiations as well as on the time between the end of the irradiation treatment and sowing of the pollen. The time interval between irradiation by X-rays and u.v. radiation can be as long as two or more hours with no very extensive decrease of the effect. For pre-treatment with u.v. radiation, the magnitude of the synergism increases as the interval between radiation treatments is extended to 60 min and then decreases to its initial value as the interval is further increased to 120 min. In the latest work of this group it was observed that irradiation of the pollen with as little as 0.5 r of X-rays followed by approximately 10,000-20,000 ergs/cm<sup>2</sup> of ultraviolet results in a definite synergistic effect. In this work, which is just being developed, the wavelength dependence of u.v. necessary to produce the effect is being determined as well as the dependence of the synergistic effect on the linear energy transfer (LET) of the ionizing radiation used. More recent studies also show that the magnitude of the effect is also dependent on the amount of solar radiation the pollen receives prior to the u.v. and X-ray treatments given in the laboratory. The potentiation of the synergism thus appears to be a photobiological phenomenon. This investigation reminds me very much of earlier experiments combining infra-red with X-rays. Initiated some years ago in co-operation

with B. P. Kaufmann (Kaufmann *et al.*, 1946) on *Drosophila* and later on with C. P. Swanson (Swanson and Hollaender, 1946) on *Tradescantia* chromosomes, these studies established quite well the synergistic effect of near infra-red radiation. Some investigators now believe that the cause is really in the far red, not the infra-red. Incidentally, this red effect will increase the X-ray damage significantly. The results of the red effect are similar to that of the u.v. and X-ray combination. This shows again that what we usually observe in our measurements is probably only a very small part of the action of ionizing radiation. Through ignorance or failure to use more sophisticated methods of radiation, we may miss some of the important initial effects of radiation. The more we investigate details, the more it appears that there is hidden from us a good part of the initial damage which the cells probably repair themselves and which we have not been able to evaluate in any quantitative manner.

Another series of experiments is concerned with protection against radiation damage by chemicals (Stapleton, 1960) and possibly some different approaches to the repair of radiation damage after exposure to radiation. The first of these projects is one in which I am especially interested, that in regard to mutation production. We reported years ago (Hollaender and Stapleton, 1955) that the mutation rate in *Aspergillus terreus* has always been inversely proportional to the survival of the organisms that have been exposed to X-rays. In a careful analysis of the survival curves, we learned that the killing curve in *A. terreus* is S-shaped where up to 10,000 r no organisms are killed. It has also been observed that one can protect against mutation production by either the removal of oxygen or by using certain types of chemicals. For all purposes, cysteamine was the most useful and most successful one. But even after this treatment, the more cells that survive, the lower the mutation rate. Inasmuch as we have no killing in *A. terreus* at doses below 10,000 r we thought we could investigate mutation rate free of the complication introduced by decreasing survival. We proceeded by simply plating a very few spores since the mutations are usually crowded out and grow more slowly than the normal colonies. We used between 20 and 50 plates, and thus only had a few colonies on each plate. By this method we found (Hollaender and McCarthy, 1959) that the mutation rate is directly proportional to the amount of radiation given and independent of the survival in the initial phase where it is 100 per cent. If we give cysteamine in the proper concentration during irradiation, the mutation rate is cut down significantly. From these results, we infer that killing of cells and mutation are not necessarily interdependent and that we can protect against mutation

production without effect on the killing rate of the radiation. This is a rather significant observation because it shows that true protection by chemicals exists against mutation production without dependence on the survival ratio.

Another project in our laboratory is the study of how chromosomal breaks can be modified by treatment after exposure to radiation. One of our associates, Sheldon Wolff (1960), has found that he is able to modify the rejoining of broken chromosomes either by inhibiting protein synthesis with chloramphenicol or by enhancing the rejoining of chromosome breaks with an energy source like adenosine triphosphate (ATP). This is possible in chromosomes since the breaks stay open after exposure to X-rays for about 30 or 40 min. Different types of treatment are possible during this period. This is a very significant field since it shows us we can manipulate irradiation damage by different methods. I am sure these studies will lead to many other interesting observations on effects of radiation on living cells.

The work of R. F. Kimball *et al.* (1959, 1960) of our laboratory has also been concerned with radiation damage. We observed in our laboratory years ago that radiation damage can be reduced very significantly by treatment after radiation. At that time we used methods which inhibited cell division but which still permitted metabolism or enzyme activity to take place. The work was done mostly in regard to survival, but later on was extended to mutation production. This work has now been verified in a very striking way by Kimball in regard to the repair of pre-mutation damage in *Paramecium*. His studies again demonstrate the ability of cells to repair a good part of damage. He observed that the number of recessive lethals and slow-growth mutations produced in *Paramecium* were reduced by a factor of two by post-irradiation treatment by either chloramphenicol, streptomycin, or caffeine and also by starvation. But again, as has been pointed out previously, the amount of mutation can be modified only if the post-treatment is begun before chromosome duplication takes place. It happens that agents which produce this post-treatment effect also delay chromosome duplication. Thus more time is made available between irradiation and duplication for repair of the damage; consequently less mutation is produced. The rate of this change in mutation is decreased by starvation and by various metabolic inhibitors. This verifies again the early observation that radiation damage is not definitely frozen but that by careful analysis and careful investigation of different factors involved, mutation production and survival can be manipulated in a very definite way. Careful analysis by Kimball pinpoints the period in which this repair can take place, i.e. especially before chromosome

duplication. This effect is not as visible after chromosome duplication, but there are some very basic questions which still have to be answered. *Paramecium* lends itself to careful investigation in this field.

All these studies establish that it is possible to manipulate living cells in regard to survival, rate of mitosis, chromosome breaks, and mutation production. If we analyse carefully what takes place in these cells and if we use modern tools, such as tritiated thymidine to detect DNA synthesis and metabolic inhibitors to influence protein and nucleic acid synthesis and energy metabolism, we can follow the steps leading from the initial absorption of the radiation energy to the final chromosome break, rejoining of chromosomes, or establishment of a mutation. It appears now that these different processes lend themselves to practical manipulation. These studies will afford us, with the cooperation of the biochemists, a much better picture of how radiation damage is initiated and how it will express itself. Such a line of investigation will also give us possible tools to study the synthesis of proteins and nucleic acids—the area where probably our greatest development will take place.

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† For further references on this subject see volume: "Radiation Protection and Recovery" (A. Hollaender, ed.), Pergamon Press, Oxford, 1960.

#### DISCUSSION

ALEXANDER: Have you the data which would explain why the doses necessary for inhibition of mitosis vary so widely from one cell to another? In mammals and in some cultures we have not seen any effects of radiation on mitosis within the first 24 hr after the exposure, even when 300 r were applied.

HOLLAENDER: There is an interesting paper in which a similar effect of irradiation on the mitotic rate in the skin cells of the mouse ear was established. Radiation doses here were greater but they were not too high. Dr. Karson very elegantly studied this problem on grasshopper neuroblasts. He observed chromosome damage in living cells. I think it could not be done so easily on mammalian tissues.



ALEXANDER: Have you data indicating that post-exposure treatment, decreasing incidence of the mutations, facilitates simultaneously the repair of the damage leading to the cell death? Is there a similarity between the process of mutation repair and that of repair of other cell damage?

HOLLAENDER: I believe that there is a difference. However, at present I cannot answer this question because I do not have the necessary experimental data. It is possible that in order to solve it, it would be necessary to work out a special method.

I believe that mutations differ from the processes of the general cell damage and I think that the processes of the initial damage in these phenomena are different.

BACQ: Did the oxygen effect occur when *Aspergillus terreus* spores were protected? For me it is important to know, whether this compound protects against oxygen or not.

HOLLAENDER: We have not yet studied it.

BACQ: Do not you think that it is an interesting field for investigation?

HOLLAENDER: Every investigation consists of many stages. I think it would be interesting to study this question. It would not be very difficult to solve it.

LEBEDINSKY: Would you not agree to apply the term "extracellular factors" to those compounds which affect the occurrence of back-mutations? And, if so, have you not tried to classify these extracellular factors?

HOLLAENDER: In our work all our attention was focused on the experiments which permit quantitative estimation. We are sure that the mutation process is affected also by extracellular factors. To study this problem it is possible to irradiate only cytoplasm but for this much greater doses should be applied. At present no precise method of studying these changes is available. We transferred nuclei into the irradiated cytoplasm, but have not as yet obtained clear-cut results. I would prefer not to speak about it now. I am sure that methods of studying the cytoplasm could be devised, which would help to study qualitative changes.

HOLMES: I would like to point out that in sperm owing to metabolic processes there is enough energy. I believe that you probably mean some *special* metabolic processes when you say that the processes of mutation repair in sperm are impossible because the metabolism is lacking.

HOLLAENDER: I should in this connection point out that metabolism in the sperm differs from that in spermatogonia. Maybe metabolism which occurs in sperm, cannot stop the processes occurring after the irradiation. However, I am not convinced that it is so. There may be alternative approaches which would make it possible to interpret these facts otherwise.

GRAY: I would like to make one remark with regard to the different effects obtained when sperm or spermatogonia were exposed. In particular, when a lack of repair in sperm is stated, is it not due to such changes, produced by irradiation of sperm and ovum, which are irreversible? But it is not of course a mutation. You have said that this is the first work on mutation repair—of course it should be continued.

I would like also to say several words about Dr. Alexander's remark. Maybe it is worthwhile to remind ourselves that it is necessary to distinguish between the effects on the incidence of mitosis and on the duration of the separate phases. I



believe that this latter effect may be seen only when irradiation is given in small doses and at a particular time. In this direction studies were made by Dr. Shirin who applied comparatively small irradiation doses between phases. As a result a delay of the next phase was observed.

HERČIK: Please explain whether you consider the effect of the fasting in your interesting observations as specific, or whether the fasting is acting in the same way as other agents in lowering the cell division rate? If it is so, then agents increasing mitotic activity should increase cell sensitivity. Is it possible to explain by these relationships the differences, sometimes very considerable, in the sensitivity of mitosis not only in different species, but within the tissues of the same species?

STRELIN: According to your data is the effect of fasting specific or is it only a factor which lowers the intensity of cell division? If it is so then is it possible, beside the activity of cell division, to establish also in an adequate manner cell sensitivity changes using those criteria which have been studied? And if these are general regularities, then could they account for differences in the radiosensitivity, including those regarding mitotic activity, not only in different animal and plant species but within the same species? Sometimes this sensitivity differs a thousand-fold.

HOLLAENDER: If I understood the questions correctly, you ask whether it is possible to explain the difference in sensitivity not only between different species but within the same species with regard to radiation effects on mitosis. I have no explanation for different radiosensitivity. I can only emphasise the fact that we have no bacteria that could be studied when irradiated with a dose of a million roentgens. They do not survive. There are hardly any bacteria that would survive it.

ERRERA: I would like to give some details pertinent to the question of metabolism in sperm. In sperm there is no protein synthesis whatsoever and metabolic activity of the respiratory systems present is directed towards ensuring the movements of the sperm. In the nucleus no metabolic processes occur.

MARKOVICH: Did you study the influence of the dose rate while studying the effect of light?

HOLLAENDER: This effect was discovered several months ago: we did not study the effect of the dose rate. To our dissatisfaction, when we collected the pollen on a misty dark day, we observed effects, different from those observed when collection was made on a sunny day. Very careful controls had to be made before consistent results were obtained. In one case we got 18 per cent, in another 12 per cent. The effect exists, but I am unable to give any details with regard to it.

POWERS: What mutations in *Aspergillus* did you record and do you know anything about their stability?

HOLLAENDER: The mutations which we recorded were morphological mutations connected with pigment formation and so forth. They are stable. During the last 15 to 20 years we have studied them very carefully and you will find a description of these phenomena in our work. But sexual process here is lacking and crossing could not be realized.

POWERS: I remember your first work and because of it I possibly got a wrong conception. Maybe the changes obtained are non-nuclear? Since sexuality is lacking, we have no test to check on it.

HOLLAENDER: It is true that this question may be solved only by crossing. In order to verify it at present the appearance of biochemical mutations and back mutations in *E. coli* are being studied.

SHABADASH: Would you state in a general form that in mammals, beside intercepting radicals when cysteamine is used, it is most important to achieve metabolic changes?

HOLLAENDER: Theoretically it is a very complicated problem.

ASTAUROV: What mutations did you record in the work with *Paramecium*? As far as I understood recessive mutations had been followed up, but these mutations would be masked by the influence of the multiploid nucleus. How were they recorded?

HOLLAENDER: In Dr. Campbell's work a special strain is used. In this case recessive mutations can easily be followed up.



# ON THE MECHANISM OF INHIBITION OF CELL DIVISION INDUCED BY IONIZING RADIATION

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## SUMMARY

The inhibition of mitotic activity lies at the very basis of the radiation effect on physiological regeneration.

The inhibition of this activity may result from:

(i) radiation damage to the synthesis of the products that are necessary for cell division:

(ii) damage to the mitotic apparatus with the blocking of mitosis even in the case where there is a sufficient quantity of the products necessary for cell division.

The biochemical disturbances are reversible, the genetic disturbances are irreversible. The cells with the damaged genetic apparatus give rise to pathological mitoses and perish during the first generation. On the basis of these assumptions a formula was elaborated for determining the index of normal mitosis as the function of time and irradiation dose. Besides the intracellular processes mentioned the mitotic activity of cells in an organism is influenced also by extracellular processes.

The report presents experimental data concerning the influence of nervous, hormonal and cytolytic factors on mitotic activity in irradiated organisms. The necessity to modify the equation is suggested so that both intracellular and extracellular factors may be taken into account.

One of the most important problems for our understanding of the living organism is the process of physiological regeneration. The normal realization of this function is based upon periodically repeated mitotic activity in the cells.

One of the most characteristic effects of radiation upon biological objects is the inhibition of the processes of physiological regeneration. The inhibition of mitotic activity lies at the very basis of this phenomenon, which has attracted considerable investigations. Their significance is not only in the fact that they enable us to study several general laws concerned with cell reactions in response to the radiation effect but also in the evidence that the experimental results obtained enable us to penetrate deeply into the very essence of the phenomenon of mitotic activity.

The most general form of the reaction arising in response to radiation

within the range we are mostly interested in is the phenomenon of mitotic inhibition. Similar inhibition may be accompanied by subsequent restoration, whilst the duration of the inhibitory time interval depends upon the number of ionizations occurring within the biochemically efficient volume. The other form of mitotic inhibition may be termed the genetic one. In its very essence it is of an irreversible character; it develops very rapidly, leads to the appearance of pathological mitoses and to destruction following a few subsequent generations. And lastly, the third form is also of a basic genetic nature, it is also of an irreversible character and develops rapidly whereby the cells are immediately destroyed. The inhibition of mitotic activity following doses exceeding those of case 1 and case 2 leads as well to the destruction of cells.

The restoration processes mentioned above are included as obligatory components of the tissue reaction to the radiation effect. When the lesions occur in a limited biochemical volume, the restoration may be of a complete and true character, i.e. it takes place at the expense of cells that have experienced the damaging effect of radiation. In the cases where genetic material is affected, the restoration is, practically speaking, but a process of compensating for the defect at the cost of cells having avoided damage. The very existence of a similar mechanism under high dose radiation in the corneal epithelium of the mouse was established by Strelin (1934, 1956).

One of the final goals of investigation in the study of the effect of radiation upon mitotic activity is the formation of equations to represent the kinetics of the populations studied; for the system composed of an extensive series of constituents statistical analysis is most convenient. As the main effect is displayed in the retardation (delay) of cell division, the aim of this kind of investigation is to determine the probability  $K$  of division and the estimation of the perished cells  $I/\tau$  per time unit in relation to time  $t$  and the radiation dose  $\Delta$ .

This type of statistical analysis is based upon a detailed elaboration of a pattern of radiation injury introduced into cell division. In its turn the pattern of the radiation injury caused to mitosis should be viewed as a sequential event following from the pattern of normal mitotic activity. For this purpose we have utilized the scheme of Watson and Crick (1953).

On the basis of this scheme the ideas dealing with radiation injury to mitosis are as follows:

It is known that the synthesis of biochemical materials used during mitosis occurs during the interkinetic period and terminates at the beginning of mitosis. The very fact that radiation inhibition of mitotic



activity may be brought about most easily by irradiation throughout the interkinetic period reveals the importance of damaged synthetic processes in the radiation inhibition of mitosis. In mentioning the synthetic processes we should indicate first DNA synthesis. Thus, ionization in the biochemical volume may lead to the appearance of a retardation in the rate of the synthetic process, this fact may be viewed as one cause of radiation damage to mitosis. At present it is hard to identify the damage to the genetic apparatus with any other definite phenomena. In any event one may observe the blocking of mitosis under conditions of sufficient DNA supply, it may also then depend upon damage to the chromosomal apparatus.

At any rate, the blocking of the processes of biochemical synthesis or damage to the genetic mechanism leads to a decrease in the intensity of the cell division process  $K$ ; this fact is clearly observed post radiation. The decrease of  $K$  is not always easily deciphered. However, suitable experimental conditions may be produced and they may provide conditions for the restriction of the reaction to definite cell areas.

Similar experimental conditions are produced when exponential cell populations are exposed to irradiation i.e. when there is not a limiting factor effecting cell propagation (insufficient nutrient material and excessive metabolic processes). In this case all phenomena relating to reaction to radiation may be considered from a practical standpoint, as intracellular effects.

The study of intracellular effects of radiation is a matter of first rate importance. It may be solved by means of statistical analysis, having estimated the possible changes of  $K$  during the process of inhibiting mitotic activity and its restoration to its unchanged form. The action of radiation may be also studied via pathological mitosis. To simplify our reasoning we supposed that there are only two reasons for inhibition of mitotic activity: (i) blocking of the synthesis of biochemical complexes required for mitotic activity and (ii) blocking of genetic mechanisms. Some of the cells in the irradiated cell population may be unchanged whereas in others the processes of biochemical synthesis are blocked; in a third group both synthetic processes and genetic activity are blocked. The blockade of genetic mechanisms occurs immediately and is of irreversible nature, and a cell with a damaged genetic mechanism perishes after a single and sole division. As has already been indicated these assumptions enable us to determine the course of inhibition of mitotic activity and its restoration. The curves obtained may be compared with the experimental data, and if they coincide they testify to the reality of the initial assumptions i.e. they may serve as material for the analysis of experimental data.

The theoretical curve giving the mitotic index of the irradiated population as a function of the time and radiation dose is presented in Fig. 1.

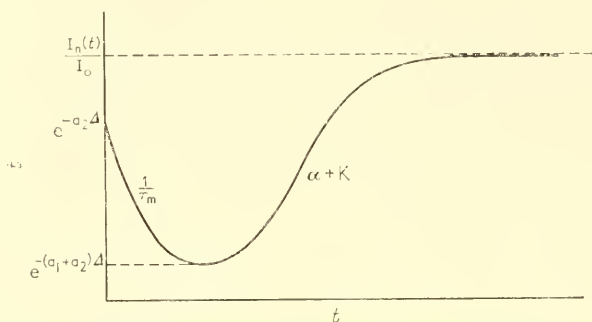


Fig. 1.—Radiation damage to mitotic activity.

The mitotic activity after radiation reduces in an exponential fashion, its period amounts to  $\tau_m$  (mitosis duration). The initial value of the mitotic index is determined by the number of cells that have avoided damage to the genetic apparatus immediately during the mitosis stage. The minimum value of the mitotic activity is estimated by the number of cells which avoided both biochemical damage and damage to genetic structures.

The intensity of restoration is determined by the value  $\alpha + K$ .

The form of the restoration curve depends upon the irradiation dose or upon the rate of cell damage (Fig. 1).

At a small dose of irradiation when the intensity of cell restoration  $\alpha$  is much greater than the intensity of cell renovation, the form of the restoration curve is near to the exponential.

The curve will have an S shape if the irradiation dose is great and cellular renovation forms the very basis of the restoration of cellular activity.

An analogous restoration curve was obtained by Powers (1955), but the ground of his considerations was somewhat different from ours. The author regarded as the object of restoration not the cell, as we did, but a certain intracellular substrate the content of which in the cell determines the intensity of cell division. Though, according to the author, the restoration rate of this substrate does not depend on irradiation dose, the probability of the restoration of the cell's mitotic activity depends (as it does in our case) on the radiation dose in view of the relationship between the quantity of damaged material inside the cell and the radiation dose.

These considerations are adequate in regard to the exponential population i.e. a particular situation where cell propagation is determined only by intracellular events on the basis of our calculations. We have used in our considerations the notion of only two intracellular phenomena: processes of synthesis and the removal of metabolites. However, we are aware that the intracellular factors for mitotic activity are not limited only by the inhibition of synthesis of the definite biochemical precursors and the removal of the resulting metabolites. Experiments show the importance of kinetics in the formation of toxic products in the irradiated cell, as was demonstrated by Ilyina and Petrov (1960). I wish to remind you that according to these authors the toxicity of mitochondria and microsomes in the small intestinal mucosa of the irradiated animals increases sharply. It is not excluded that the transfer of these toxins towards the nucleus affects the mitotic processes.

Besides the toxic products arising in mitochondria and microsomes in the irradiated cells there are other intercellular factors changing the mitotic activity. This is not enough. Actually we must admit the existence of a great number of extracellular factors that are active in cell division, even in the case when in the purposes of simplifying one chooses the simplest biological object—the corneal epithelium devoid of a blood supply system.

As to this and some other objects it is easy to demonstrate that the notion about the real events in the cellular population may be created only by taking into account the existence of extracellular factors.

We supposed above that the probability of cell division in the basal layer per time unit depended upon the concentration of the metabolites in the media surrounding the cell. In the first approximation we may assume that we should speak chiefly about the DNA synthesized in cells.

The experimental data of Skovronsky, Fradkin and Borisova (1961), may serve as new evidence in favour of this view. They showed that, against the background of stimulating nucleic acid synthesis and inhibition of protein synthesis with the aid of small concentrations of levomycin (1 mg/ml), the number of cells capable of division in irradiated ( $^{60}\text{Co}$ ) suspensions of *E. coli* increased (Fig. 2). And on the contrary, the number of dividing cells is considerably less against the background of almost total blockade of protein and inhibition of nucleic acid synthesis initiated by the very same antibiotic at large concentrations (60 mg/ml, Fig. 3).

There are other instances of inhibition of DNA synthesis caused by radiation and of the role of this inhibition in the destruction of processes

of mitotic activity. However, observing respective data one may see that there is no direct proportionality between the disturbance of DNA synthesis and the radiation dose.

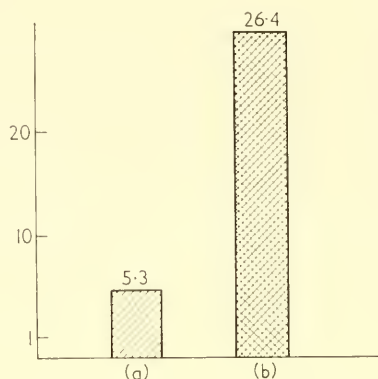


Fig. 2.—The number of dividing cells in *E. coli* cultures against the background of the preliminary stimulation of nucleic acid synthesis and a certain inhibition of protein synthesis (in percentage of un-irradiated control):

- (a) irradiation under normal growth conditions
- (b) irradiation against the background of a stimulated nucleic acid synthesis.

This view may be supported by the experimental data of Libinson and Konstantinova (1960).

The authors worked with a truly non-exponential system and determined the amount of nucleic acid in the liver and bone-marrow (Schmidt and Thannhauser's method modified by Davidson) and the rate of incorporation of  $^{32}\text{P}$  4 hr after the introduction of  $60\text{ }\mu\text{C}$  of  $\text{Na}_2\text{H}^{32}\text{PO}_4$  solution per kg of animal. The average content of nucleic acid in a single cell of the tissues was found by counting the number of cellular nuclei per gramme of tissue. Possibly, the most valuable form of experiment is repeated irradiation with small doses.

Libinson and Konstantinova found that exposure of animals at a rate of 30 r per day led to some reduction (24 per cent of DNA-P per g of liver tissue) in the early time intervals following the beginning of the experiment (the total dose was 420 r). Then the content of DNA-P increased and reached the initial values (the total dose was 2,490 r).

The authors have also showed that the incorporation of  $^{32}\text{P}$  into the nucleic acids as determined by the state of the synthetic processes for these substances in the liver shows an increase of specific activity of 2.2 for DNA, the total dose being 420 r (30 r for 14 days). After 900 r the specific activity of the DNA was 26.4 per cent of the control. At

doses of 1,400 r and 1,920 r the decrease of specific activity in DNA was less pronounced.

It is of interest that a dose of 630 r caused a marked reduction in the number of cell nuclei (19 per cent). The next exposure leads to the restoration or even increase in nuclear number.

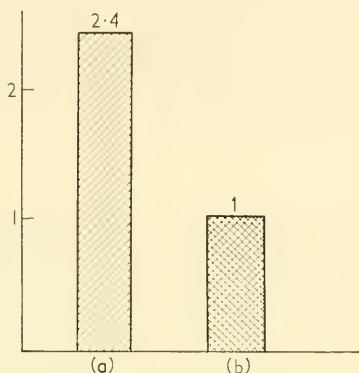


Fig. 3.—The number of dividing *E. coli* cells in irradiated cultures against the background of a blocking of protein synthesis and inhibition of nucleic acid synthesis (in percentage of unirradiated control).

(a) irradiation under normal growth conditions;

(b) irradiation against the background of a block in protein synthesis and inhibition of nucleic acid synthesis.

Very similar data were obtained in studies of nucleic acid changes in bone-marrow. At first, repeated exposures to doses of 30 r failed to produce a pronounced change in the nucleic acid content of bone-marrow. The total dose of 900 r led to considerable decrease of PNA-P and DNA-P, the latter increased when the dose was 2,500 r. These data, of course, do not deny the prime importance of DNA synthesis for mitotic activity.

One of the reasons for the absence of relationship between the change of the DNA content and the exposure dose may be the fact that the exposure of the entire organism involves rather powerful extracellular factors. Indeed, the present day science has accumulated a sufficient number of facts demonstrating the controlling influences of nervous and humoral effects upon DNA synthesis and these materials require certain corrections to the cited scheme of distribution.

There are several experiments in favour of this point. We shall cite only one taken from Pozdniakov's work who showed the increase of DNA decomposition in connective tissue under the effect of humoral and nervous influences (Lebedinsky *et al.*, 1960).

Plate I shows the normal fluorescence of rabbit conjunctiva (acridine



orange stain); Plate II shows the same object after the stimulation of the elements of the first trigeminal branch, the disintegration of nucleic acid being seen distinctly. Plate III shows a more strongly pronounced picture of the DNA disintegration which appears after the destruction of Gasserian ganglia.

No doubt, extracellular factors do exist and play a definite role in DNA changes arising during exposure of biological objects. They should play a definite role in the reactions to exposure. This ensues from the experiments of Kuzin and Budilova (1953, 1954) who showed that the changes of nucleic acids in the spleen vary in relation to whether exposure was local or whether the animal's head was subjected to irradiation.

Similar experiments do not yet show that disturbances of DNA synthesis arising in the cell as a secondary matter (under the effect of extracellular factors) are related to mitotic activity. However, special experiments have been carried out which showed that it was undoubtedly so, for instance, experiments with adrenaline inhibiting mitosis (Aloy, 1956; Strelin, 1954). According to our experiments deoxyeortioesterone acetate (DOCA) has a definite effect as well.

Figure 4 shows changes in the relative mitotic activity of the corneal

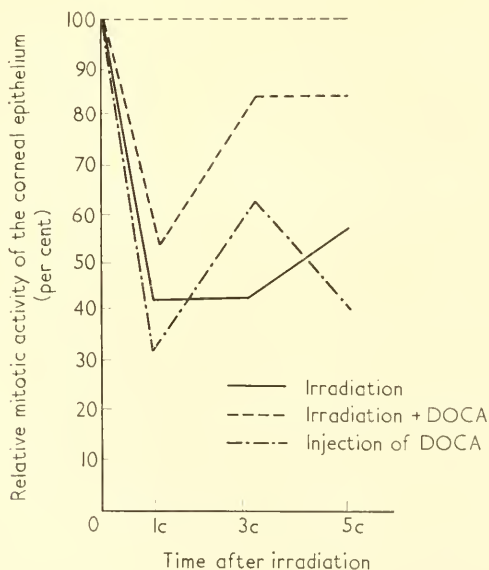


Fig. 4.—The effect of DOCA on the mitotic activity of the corneal epithelium of the mouse.

100 per cent is taken as the norm.



Plate I.—Normal loose conjunctival tissue of the rabbit. Red fluorescence of cytoplasm and yellow-green fluorescence of fibroblast nuclei. Ocular 6, objective 8.

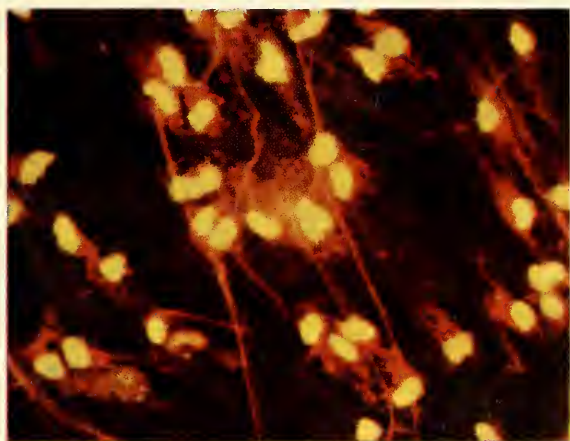


Plate II.—Loose conjunctival tissue of the rabbit. The second day after the transection of the first trigeminus branch. The fluorescence of nuclei and cytoplasm of the fibroblasts is brighter than normal. Ocular 8, objective 8.

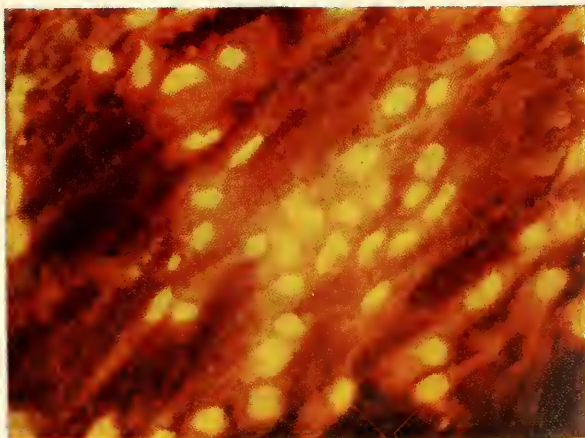
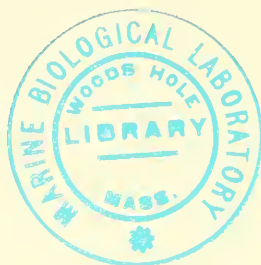


Plate III.—Loose conjunctival tissue of the rabbit. The second day after the transection of the Gasserian ganglion. The fluorescence of nuclei and cytoplasm of the fibroblasts is extremely bright; the fluorescence colour shifts to tones more red than normal. Ocular 8, objective 8.



epithelium of the mouse under conditions of DOCA subcutaneous injection (1.10 to 4 mg). The third curve from the top characterizes this inhibiting action. Combining the DOCA injection with irradiation, it is possible to observe a stimulating effect (the first curve).

It is still necessary to show that these extracellular factors participate in the changes of DNA synthesis in irradiated animals. In fact, evidence for this is included in the experiments of Kuzin and Budilova.

Taking into account the role of extracellular factors for statistical purposes is one of the most important tasks in formulating a theory of radiation damage of mitosis. So far, however, the very concept of extracellular factors is far from being deciphered. One should not believe that extracellular factors are represented solely by nervous and humoral influences. Actually, the events are much more complicated. The cytolytic systems of the organism may effect the fate of cells exposed to radiation. They may play a certain role in the processes of restoration facilitating the release of the cell population from the effect of the perished cells. It is well known that after radiation the situation is unfavourable: along with numerous perished cells, the cytolytic activity of the tissue fluid decreases (Zaretskaya, 1960).

Figure 5(a) shows the dependence of the number of lysed rabbit bone-marrow cells upon the exposure time of normal rabbit aqueous humour, Fig. 5(b) shows the same process under the influence of the aqueous humour in irradiated animals: the marked decrease of the lysing capability of the serum, especially on the 5th and 7th days after irradiation is seen distinctly from this figure.

It is doubtful whether the cited data cover all the possible ideas concerned with the extracellular factors affecting the mitotic activity of the tissue of exposed animals. So we should think about the complex system of nervous and humoral disturbances of the regulatory mechanisms to explain the data of Gruzdev (1960, unpublished) who showed in mice exposed to total radiation (750 r) the disappearance of the typical diurnal rhythm of mitosis (Prof. Horizontov's laboratory). We attempted to give the schematic picture of the possible additional cellular and extra cellular factors capable of influencing the mitotic activity of the normal (left) and irradiated (right) cell (Fig. 6). These are hormones, mediators of the respective nervous influences, cytometric factors and some others.

It stands to reason that this scheme is inadequate for the statistical analysis of the system undergoing the extracellular effects. Indeed, specific ideas about the essence of the extracellular effects are greatly limited. The studies of a number of these extracellular effects reveal their mainly inhibiting action on mitosis. In this case most of them

seem to be represented by a coefficient less than 1 multiplied by the probability  $K$  of division. However the temporal and dose dependence of this factor have not yet been studied. Most of the extracellular factors exert their influence upon metabolism. Among them there exist the

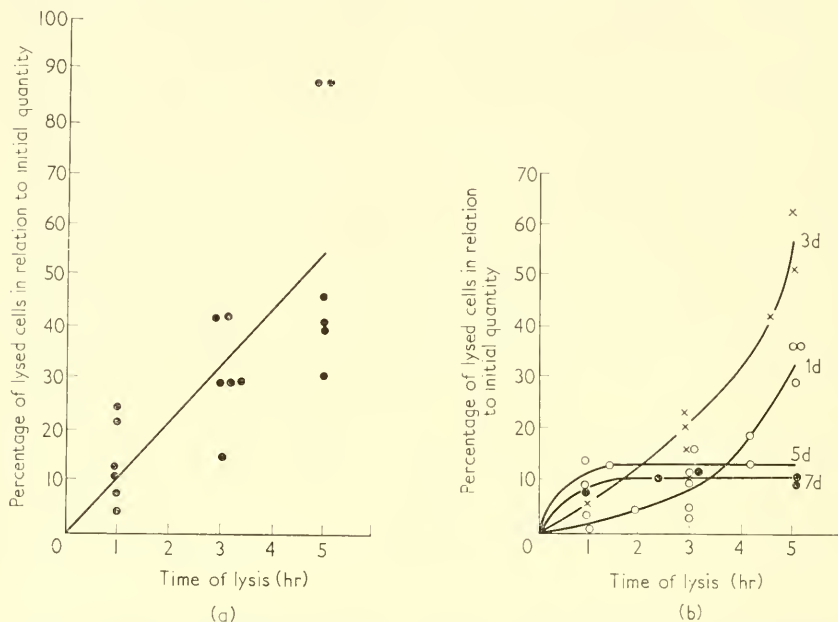


Fig. 5.—Dependence of the number of lysed bone-marrow cells upon the exposure time of rabbit aqueous humour on them:

(a) before irradiation.

(b) after irradiation.

factors that contribute to the liquidation of radiation injury before the start of the mitotic stage by affecting the intensity metabolism. Therefore the apparent significance of radiation biological effectiveness will decrease. Other things being equal this effect is seen most clearly in the cells with a longer duration of interkinesis. It is highly probable that a population of this kind may show with particular clarity the apparent decrease of biological effectiveness under the corresponding extracellular influences. From this point of view the mainly inhibiting action of extracellular factors upon mitotic activity is worthy of attention. Under their effect the intermitosis as it were lengthens, and thus the temporal possibilities for restoration from damage increase.

The mathematical treatment of observations accumulated in studying biological material and its phenomena necessitates certain approximations. We are forced to approximate consciously, not forgetting one



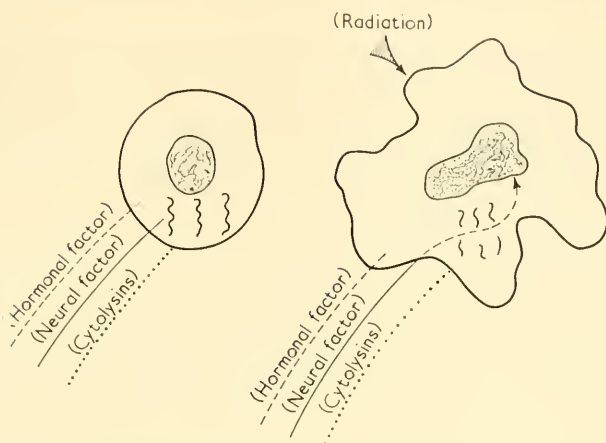


Fig. 6.—A scheme of possible additional cellular and extracellular effects.

obligatory condition: while producing one or other type of equation we should not forget the existence of processes that play a principle role in our understanding of these phenomena. Their number in our case includes several additional intracellular and extracellular factors, the latter being especially interesting for us. For this purpose it is necessary firstly to study in detail the significance and the character of their inclusion into the radiation reaction of the organism and the dose dependence.

That is, we raise with all possible sharpness the question about the rapprochement of common physiology and radiobiology.

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## DISCUSSION

ARDASHNIKOV: Have you got experimental curves that could be compared to the calculated theoretical one?

LEBEDINSKY: Yes, we have. Our curves fit the experimental data. Even with small irradiation doses the probability of recovery is small. Practically any family of curves may be obtained, fitting in with experimental data.

ARDASHNIKOV: I understand that any curve family can be obtained, but it is important that the experimental curve should coincide with the theoretical one.

MEISSEL: I would like to know for how long after the manipulation on the nervous system elements there occur the changes in the character of the nucleic acids' fluorescence which you have established? In our experiments this phenomenon was not observed, even when more highly organized systems were used.

LEBEDINSKY: For 24 to 48 hr. There is an old fluorescence test, known I believe for the last 20 or 30 years, and used also by doctors. The fluorescence of the corneal epithelium manifests itself within 10 to 15 min following surgery on these nervous elements.

MEISSEL: But in the case when fluorescein is used it is the membrane permeability changes that account for the effect, whereas here the effect should be explained in terms of the considerable changes of the cell metabolism connected with the nucleic acid changes.

LEBEDINSKY: At the time when a surface defect is produced, there are no permeability changes as yet. For example, you cannot detect penetration of the fluorescein into the anterior chamber of the eye. At the same time the test, results of which have been reported, testifies to a considerable change in the intermediary conjunctival membrane of the eye, induced by nervous irritation.

# THE BIOCHEMICAL MECHANISM OF THE DISTURBANCE OF CELL DIVISION BY RADIATION

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## SUMMARY

X-irradiation of fully grown leaves of *Vicia faba*, causes almost immediate activation of oxidative enzymes of the polyphenoloxidase and peroxidase type. The products of oxidation of tyrosine (or similar substances) increase. These block DNA synthesis and thus prevent cells entering mitosis.

The appearance of "antimitotics" was demonstrated both by post-irradiation observation of the remote inhibition of mitosis in the points of growth (which were screened during the irradiation) and also by extraction of the "antimitotics" from the irradiated leaves, their purification and testing on un-irradiated roots of *Vicia faba*.

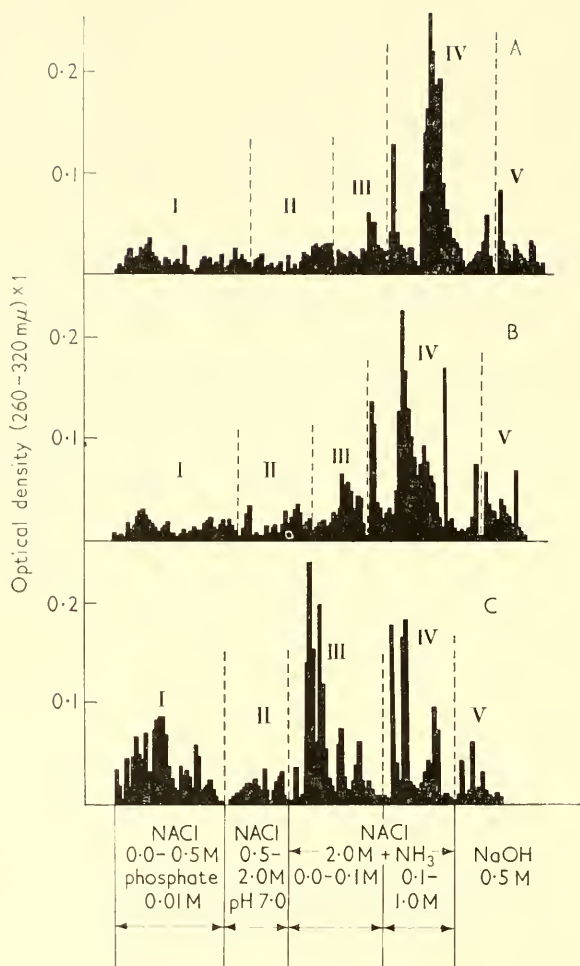
It was shown by model experiments, that low molecular weight products of the enzymatic oxidation of tyrosine possess antimitotic activity, i.e. the capacity to combine with highly-polymerized DNA and to protect DNA *in vitro* against radiation-induced depolymerization.

There has been much radiobiological literature devoted to the specially important problems of the direct action of radiation on the DNA of the cell nucleus and its indirect influence on the inhibition of cell division through altered metabolic processes in the cytoplasm.

An analysis of this problem was given by Gray (1956) at a radiobiology symposium. Facts and considerations presented by him and by Alexander, together with later results, lead us to draw the conclusion that processes going on in the cytoplasm of irradiated cells play a great part in the inhibition of cell division by radiation.

Shabadash's (1960) investigations carried out in our laboratory, showed considerable shifts of the ribonucleoproteins of cell mitochondria (the isoelectric point) within one minute of the animals being exposed to radiation at a dose of 1,000 roentgens. This phenomenon undoubtedly leads to an alteration of the oxidative processes regulated by these cytoplasm structures.

On the other hand Strajevskaya and Struchkov, while endeavouring to reveal physico-chemical changes in DNA isolated immediately after the animals had been irradiated, found that this substance was rather stable when the influence of cytoplasmic DNase, activated by the radiation, was completely eliminated. The results of measurements of the



*Chromatographic Representation of the Fractionation of Rat Thymus DNA*  
(in per cent from  $\Sigma$  difference in the wave-length 260 and 320  $m\mu$ )

Chromato-grams <sup>†</sup>	0 <sup>‡</sup>	N N fractions					return (%) from the initial
		I	II	III	IV	V	
A	7.3	13.6 ± 0.8	8.1 ± 0.9	10.3 ± 0.8	51.6 ± 2.4	9.1 ± 0.3	95 ± 2
B	5.5	12.0 ± 0.7	9.0 ± 0.5	12.1 ± 1.2	51.0 ± 0.5	10.4 ± 1.3	92.2 ± 0.3
C	5.7	29 ± 2	5.0 ± 0.3	35.3 ± 2.5	21.0 ± 1.2	3.9 ± 0.2	97.5 ± 2.2

<sup>†</sup> Average data from two chromatograms.

<sup>‡</sup> The fraction which is eluted by 0.01 m phosphate buffer pH 7.0 during washing the columns after DNA was put on.

Fig. 1.—Polymerization spectrum of DNA isolated from rat thymus.

A, DNA from unirradiated rat.

B, irrad. 1,000 r *in vivo* (10 min after irradiation).

C, irrad. 1,000 r *in vivo* (24 hr after irradiation).

TABLE I. *DNA from irradiated organs (1,000) and unirradiated rats.*

		Viscosity DNA $\eta_{sp}/C$		
Dose and time after irradiation		Thymus	Organs of rats Spleen	Liver
Unirradiated		97 $\pm$ 7	111 $\pm$ 6	83 $\pm$ 3
1,000 r	10 min	95 $\pm$ 6	109 $\pm$ 8	79 $\pm$ 5
	1 hr	—	106 $\pm$ 4	71 $\pm$ 2
	2 hr	89 $\pm$ 4	109 $\pm$ 6	83 $\pm$ 3
	4 hr	54 $\pm$ 6	106 $\pm$ 4	78 $\pm$ 4
	24 hr	20 $\pm$ 2	98 $\pm$ 1	82 $\pm$ 6

DNA concentration for the viscosity measurement was 0.004 per cent (average data from 3 to 5 experiments).

viscosity of DNA, isolated by the phenol method, are presented in Table I, and its polymerization spectrum, obtained after chromatography on Ecteola-cellulose, is given in Fig. 1. These data illustrate very well the relative stability of the DNA molecule immediately after irradiation.

The change of viscosity of DNA observed in thymus cells four hours after irradiation appears to be the consequence of the activation of the DNase in the cytoplasm of the exposed cells.

The disturbance of metabolic processes plays a special part in stopping cell division after irradiation. This is due to the distant effect of radiation on cell mitosis.

Many papers in which the distant effect of radiation on the mitotic index has been shown for animal organisms have been published, for instance by Shmidt (1948); Strelin (1950); Rode (1950); Grayevsky and Shapiro (1958); Alexandrov (1957) and Shapiro (1958).

The complexity of the neurohumoral regulatory system, of mitotic activity in the animal organism, however, makes the biochemical analysis of the phenomenon difficult. We thought it might be interesting, therefore, to trace the existence of the distant effect of radiation on mitosis for plants and to try to investigate the biochemical mechanisms, on which it is based.

Krukova and Kuzin (1960) showed that if a fully-grown leaf of *Vicia faba* was irradiated, (the rest of the plant being well screened), then, one or two days after irradiation, it is possible to observe a sharp decrease in the mitotic index. This was observed both in the apical point of growth and in the meristem of the root tip, as the results given in Fig. 2, show.

Plants placed in the same lead chamber as the experimental ones (with the leaf outside the chamber) were used for control; thus we



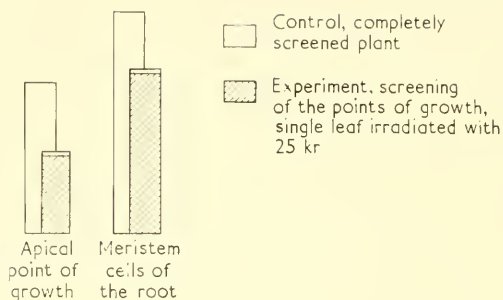


Fig. 2.—The decrease in the mitotic index one or two days after irradiation of a single leaf of *Vicia faba*. The data of 6 experiments have been averaged.

eliminated any possible slight effect of scattered radiation in the chamber.

A distant decrease of mitotic index in the points of growth lead to a 60 per cent inhibition of growth and development of the whole plant as compared with the control plants.

It was suggested that the radiation disturbed the balance of metabolic processes in the leaf, so that the concentration of certain metabolites, probably present in small quantities normally, greatly increased. We thought that these metabolites normally acted only inside a leaf cell, inhibiting cell division, but that, after irradiation the excess rushed together with the other assimilates to points of growth and there demonstrated their antimitotic activity. In order to check this suggestion, the leaf was immediately removed after local irradiation. A corresponding non-irradiated leaf was removed from the control plant. No inhibition of mitotic activity at the points of growth was observed. When the exposed leaf was removed 4 hr after irradiation slight inhibition of mitosis in the apical point of growth was observed, but none in the roots. The removal of the leaves after 24 hr already had no effect and the plants showed great inhibition of mitosis after 2 days both in the apical points of growth and in root meristem, as shown in Table II.

We attempted to extract the active substances from the leaves. Normal and irradiated leaves (20,000 r) were frozen in liquid nitrogen, pounded together with pure quartz sand and extracted with five volumes of water for 24 hr at 0°C. The extracts were filtered and roots of four-day-old normal sprouts of *Vicia faba* were immersed in this medium for 24 hr.

For control, other roots were immersed in water from the tap. After 20 hr the roots were fixed and the mitotic index determined. The results are given in Table III. The data show that extracts from

TABLE II. *Mitotic index in the point of growth on the removal of locally irradiated leaves at different periods of time after irradiation with a dose of 10 kr (from 3,000 cells)*

Conditions	Time of leaf removal after irradiation	Apical points of growth		Root tips	
		% mitoses	% control	% mitoses	% control
Control	immediately	5.7 $\pm$ 0.4	103.5	8.0 $\pm$ 0.7	111.0
Irradiated leaves		5.9 $\pm$ 0.4		8.9 $\pm$ 0.7	
Control	4 hr	5.5 $\pm$ 0.4	62.8	7.3 $\pm$ 0.6	86.3
Irradiated leaves		3.4 $\pm$ 0.3		6.3 $\pm$ 0.6	
Control	24 hr	5.4 $\pm$ 0.4	37.0	7.8 $\pm$ 0.5	60.2
Irradiated leaves		2.0 $\pm$ 0.2		4.7 $\pm$ 0.5	

normally grown leaves (where the cell division is inhibited) decreased the mitotic index in the meristem tissue of roots. But this effect was far more pronounced when extracts from irradiated leaves were used. It confirmed our suggestion that substances inhibiting mitosis are normally present in a fully grown plant leaf but that their formation is sharply accelerated by irradiation.

We investigated the radiosensitivity of this activation process, the

TABLE III. *Mitotic index in the roots of Vicia faba fed on extracts from bean leaves for 24 hr (average from 5 experiments).*

Extracts	Number of cells		Mitotic index
	general	dividing	
Control (H <sub>2</sub> O)	1,102	76	6.9 $\pm$ 0.7
Extract from unirradiated leaves	979	56	5.7 $\pm$ 0.7
Extract from leaves irradiated with 10 kr	1,000	17	1.7 $\pm$ 0.4

experiment was repeated at various dose rates, from 10 r to 25,000 r. The inhibiting effect on mitosis of such extracts is shown in Fig. 3.

We can see that 100 r irradiation already causes a certain increase of antimitotic substances in the exposed leaf. The amount continues to increase up to 500 r but after that our method failed to reveal a further increase until a dose of 25,000 r. The appearance of antimitotics in fully grown leaves is not only characteristic for *Vicia faba*. Experiments

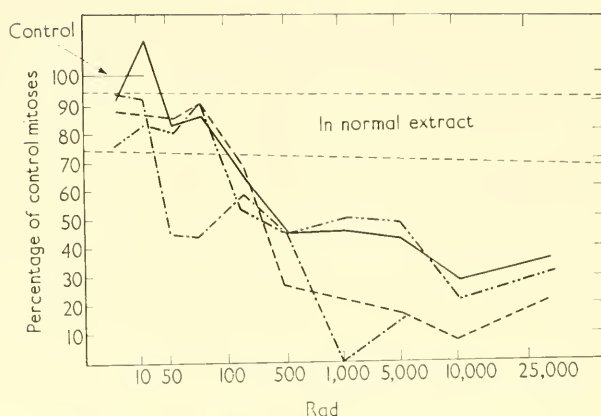


Fig. 3.—Percentage mitoses in *Vicia faba* roots immersed (for 24 hr) in water extracts from irradiated and normal leaves.

carried out with 8 different plants gave similar results. Figure 4 gives the data from this series of experiments.

What metabolic changes take place in irradiated leaves which lead

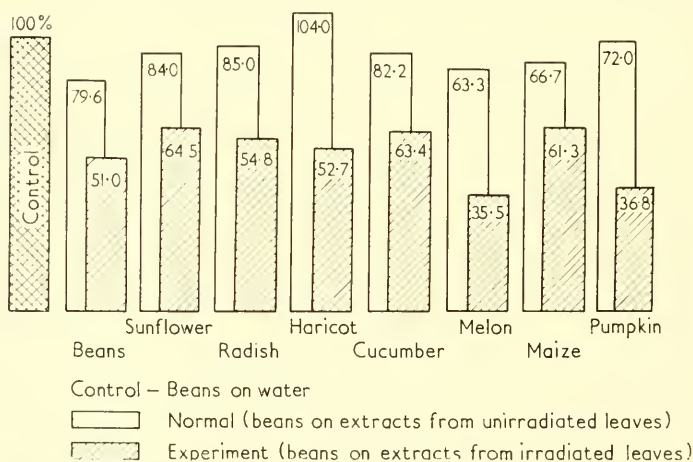


Fig. 4.—Mitoses in the roots of *Vicia faba* treated for 24 hr with extracts from irradiated (25 kr) and unirradiated leaves of different plants.

to the increase in antimetabolic substances. Kopilov's investigations showed that extracts of irradiated leaves were far more quickly oxidized by atmospheric oxygen than extracts of normal leaves. The results of a series of experiments are given in Fig. 5. The intensity of oxygen absorption by homogenates of freshly rubbed tissues of irradiated and

non-irradiated leaves was also measured by Warburg's manometric method.

Much quicker darkening of the extracts of irradiated leaves compared with normal lead us to suggest an intensification of the oxidation of

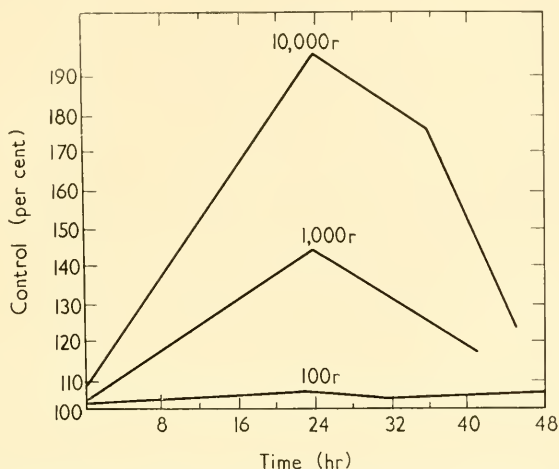


Fig. 5.—Oxidation of homogenated or irradiated leaves as a function of the dose and time after irradiation.

phenolic components. The results of the investigation of the oxidation of different phenols by extracts of irradiated and non-irradiated leaves are shown in Table IV. The data obtained showed a considerable increase in the enzymatic oxidation of tyrosine.

TABLE IV. *Intensity of oxidation of different phenols by extracts of irradiated leaves.*

Phenol	% Control
Tyrosine	220
Pyrocatechol	139
Pyrogallol	107
Hydroquinone	92

An investigation of peroxidases in the leaves of young maize plants (six-day-old sprouts), was made by Berezina, who used Boyarkin's method (1951) with benzidine, and found the results shown in Table V, which also clearly show an activation of these enzymes 1.5 hr after irradiation with 40,000 r.

TABLE V. *Peroxidase activity in maize root and leaves.*

Conditions	Leaves				Roots		
	1	2	3	4	1	2	3
Control	5.1	6.0	5.2	6.0	10.4	6.0	8.9
Irradiated (1.5 hr after 40,000 r)	7.1	9.7	6.6	8.9	16.4	11.0	11.1
Per cent increase of activity	39	61	26	48	57	89	25

These observations gave us the idea that products of enzymatic oxidation of phenols and possibly first of all, tyrosine, are substances which slow down cell division after irradiation. This suggestion was in a good agreement with the following experimental facts:

1. After the removal of proteins, which coagulate on heating, extracts of irradiated leaves preserved their antimitotic activity.
2. After dialysis of protein-free filtrates antimitotic activity was observed in the fraction that diffused through the collodion membrane. This is evidence for the low molecular weight of the antimitotics investigated.
3. When protein-free filtrates were chromatographed on alumina the antimitotics were absorbed completely. The filtrates were quite inactive. Many diphenols, semi-quinones and quinones are strongly absorbed to alumina.
4. By fractional elution of the absorbed substances with a weakly alkaline solution we succeeded in obtaining a fraction from extracts of irradiated leaves that showed antimitotic activity from extracts of control leaves, almost without activity.
5. The ultraviolet absorption spectrum showed a distinct maximum at  $280\text{ m}\mu$  in the fraction from irradiated leaves as may be seen from the curves given in Fig. 6.

The determination of polyphenols by means of their reaction with the iron tartrate reagent showed an increase in quantity in extracts from irradiated leaves. If the content of the quinonoid products of oxidation of tyrosine actually increase, then they should behave as strong oxidizing agents like peroxides. The experimental data are given in Table VI.

Our conclusion about the important role of quinonoid products of the enzymatic oxidation of tyrosine in inhibiting mitosis under the influence of ionizing radiation was proved by model experiments. We oxidized dilute solutions of tyrosine in the presence of a purified pre-



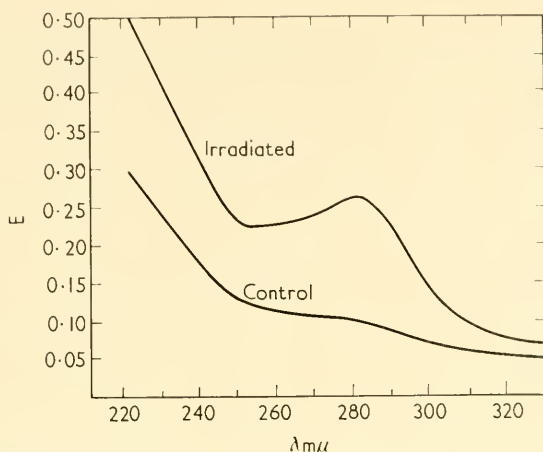


Fig. 6.—Ultraviolet absorption spectrum of an active fraction isolated from irradiated *Vicia faba* leaves.

paration of tyrosinase (polyphenoloxidase) extracted from potato tubers. The dark brown solutions containing polymeric melanins and semi-products of the oxidation of tyrosine were filtered through a collodion semi-permeable membrane. The melanins that did not penetrate the membrane were further purified by dialysis. All fractions were tested for ability to inhibit mitosis in roots of *Vicia faba*. The experimental data show that neither tyrosine nor previously dialysed melanins exhibited antimitotic activity while the low molecular weight products of the enzymatic oxidation of tyrosine sharply inhibited the entrance of cells into mitosis.

This antimitotic action of such low molecular weight products was indirectly demonstrated also by V. Kopilov and Jone-Che in experiments with young growing mice. The mice were retarded in growth,

TABLE VI. *Determination of quinone-like substances in extracts from normal and irradiated (10,000 r) Vicia faba leaves.*

	Time after irradiation	Extracts	
		normal	irradiated
1	20 min	0.03 ± 0.01	0.10 ± 0.02
	24 hr	0.04 ± 0.01	0.16 ± 0.03
2	20 min	0.03 ± 0.01	0.08 ± 0.02
	24 hr	0.03 ± 0.01	0.15 ± 0.03
33	20 min	0.04 ± 0.02	0.09 ± 0.02

both after injection of artificially obtained preparations (see Fig. 7) and after injection of purified protein-free extracts from irradiated leaves (see Fig. 8).

The mechanism of action of the products of oxidation of tyrosine on

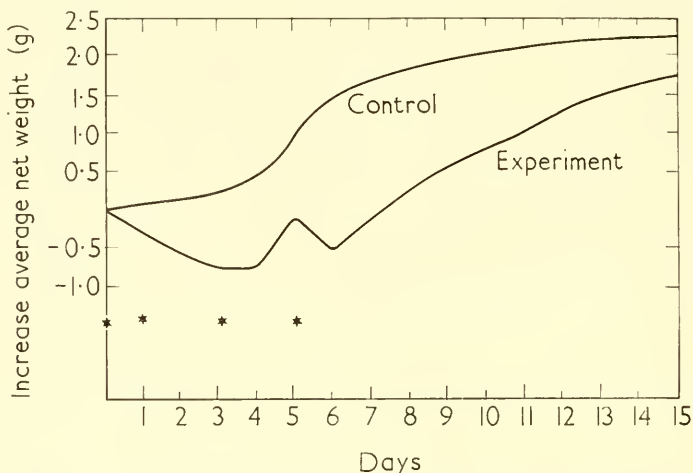


Fig. 7.—Weight curves of young mice.\* Day of injection of tyrosine oxidation product

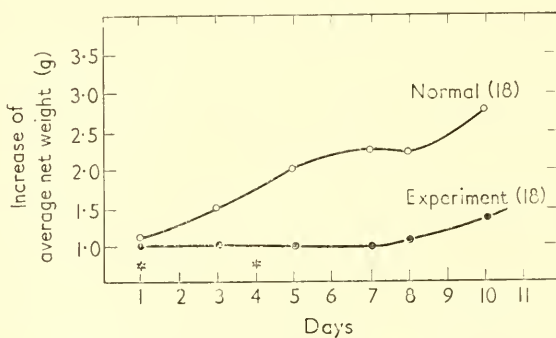


Fig. 8.—Weight curves of young mice after subcutaneous injection of purified extract from irradiated and normal leaves.\* Day of injection.

the cells nucleus is unknown but we thought it probable that it was an interaction between these chemically active substances and DNA.

The easy condensation of quinones with substances containing amino groups is well known. Quinones condensing with adenosine, guanosine or cytidine groups in DNA could considerably alter the reactivity of the DNA molecule as a whole. If such a reaction took place we could

expect a protective action against ionizing radiation. This assumption was checked in an experiment by Struchkov and Gorkina. They used a highly polymerized DNA (with a molecular weight of 8 to 10 million) extracted from calf thymus by the phenol method. Previous studies

TABLE VII. *Melanin protective effect on calf thymus DNA in vitro.*

Substance investigated	Values
	$\eta_{sp}/C$ 0.002 % p.p. DNA
1. DNA (unirradiated)	100 + 2.0 %
2. DNA + 5,000 r	40 + 2.5 %
3. DNA + initial melanin	100 + 0.5 %
4. DNA + dialysed melanin	52 + 3 %
5. DNA + filtrate of initial melanin	100 + 2.5 %
6. DNA + tyrosine	35 + 3.5 %
7. DNA + tyrosinase	52 + 1.2 %

with an 0.004 per cent solutions of such DNA showed that, even at a dose of 500 r there is definitely a fall of the viscosity immediately after irradiation. Experimental results with the DNA protective compounds which interested us are shown in Table VII. We see that neither tyrosine nor melanins, after being dialysed, prevented DNA from depolymerizing, while low molecular weight products of enzymatic oxidation of tyrosine protect DNA completely (under these conditions) from radiation action.

This protection is rather specific. For example, cysteamine, the universal protecting substance, was not protective under some conditions.

In the light of these investigations we suggest the following course of events in an irradiated cell. The energy absorbed by the cytoplasmic mitochondria effects small physico-chemical shifts in the superstructures of these cell organelles, and the co-ordinated action of enzymes in these organelles is thus disturbed. Polyphenoloxidase activity is released. This enzyme release could be closely related to an inhibition of oxidative phosphorylation. The products of enzymatic oxidation of tyrosine (or substances like tyrosine) of quinonoid or semiquinone-like structure, in abnormally high concentration and having the properties of free radicals, diffuse to a cell nucleus, where they form unstable products with DNA. The blocking of DNA prevents the passage of the cell into mitosis, and that leads to a decrease in the mitotic index.

Similar processes are possible, for animal organisms where they can explain the observed changes in tyrosine metabolism (Bak, 1955), the role of the adrenals in distant effects (Strelin, 1950; Boyarkin, 1951) and

the correct mechanism for the protective action of such polyphenols as for example oxytyramine or propylgallate (Gorodetsky *et al.*, 1960).

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#### DISCUSSION

ALEXANDER: What experiments did you perform with quinones and with DNA? It occurs to me that the effects of quinones on mitosis might be due to cell reactivity. We have never obtained any data indicating that quinones are effective *in vitro*.

KUZIN: Discussing the possible role of the oxidation half-products of the tyrosine in these phenomena I want once again to refer to two groups of facts. On the one hand artificially obtained products of the enzymatic oxidation of tyrosine greatly inhibit mitosis and decrease the root's mitotic index: on the other hand they have a protective effect on highly polymerized DNA.

SHEKHTMAN: The data you have presented are very interesting, but this question arises from them. If these products inhibiting mitosis are formed in the cytoplasm, then how do you explain data pointing to an extremely high radiosensitivity for the nucleus and the great difference in radiosensitivity existing between nucleus and cytoplasm, as demonstrated by Rodgers, Ulrich, Astaurov and other investigators? Maybe it holds true for plants, whereas in embryonic cells is it not true?

KUZIN: In our experiments plant material was used. I think however that we are justified in believing that the general mechanisms of radiation effects have much in common; only, in the animal body neurohumoral influences are obscuring the picture somewhat.

BACQ: I believe that your choice of plants for the preliminary study is quite correct. I worked for many years with quinones and know that adrenochrome is one of the very strong mitotic poisons. Lettre used it, too, with tissue cultures. It is of interest to note also, that cysteamine is an excellent oxidase inhibitor. Your data are in good agreement with Dr. Gray's ideas, since it is the tyrosinase which requires molecular oxygen. But the trouble is that in mammalian cells there is no tyrosinase and polyphenol oxidase, although the mitoses there are inhibited just the same.

KUZIN: I quite agree with you. I cannot say from my own experiments how matters stand for animal cells. But in the literature, for example in Dr.

Protassova's papers, data may be found pointing to a considerable disturbance of the tyrosine metabolism in the irradiated animals. In the animal body other enzymatic systems may be present, but the process of their activation and the formation of the quinone-like tyrosine oxidation products may be similar to those observed in plants.

PARIBOK: Did you observe in your interesting investigations chromosomal aberrations induced by substances extracted from the irradiated leaves, that is to say, was there any genetic damage other than mitotic inhibition?

KUZIN: This is a question we are very interested in and at present data are being accumulated which we hope will enable us to answer this question with some degree of certainty.

PASSYNSKY: You have reported very interesting data. Are not disturbances in tyrosine metabolism particularly important? It seems highly probable, as you have pointed out, that melanine formation plays some role here. We, in our work with Dr. Budnitskaia and Dr. Virovets, have observed considerable activation of polyphenol-oxidases following irradiation. An increase in oxidation processes takes place, caused probably not by changes in enzymatic activity but by changes occurring in intracellular membranes. Any explanation of radiobiological effects should start from an initial damage to a few molecules only. If we say that the level of the oxidation-reduction processes is changing, these changes should be caused by some preceding changes due to damage to a few molecules. The fact is that the activation of such enzymes as polyphenoloxidase or peroxidase is probably due to the damage to cellular membranes demonstrated experimentally.

POLLARD: Did you test these substances on bacteria?

KUZIN: At present we have only tested the enzymatic oxidation products of the tyrosine on bacteria.





# THE ROLE OF CELLULAR DAMAGE IN THE MAMMALIAN RADIATION SYNDROME

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## SUMMARY

The mass destruction of the cells of haematopoietic organs and of the intestine is one of the most important causes of death in an irradiated animal. The death of these cells is only observed in directly irradiated tissues not connected with mitosis and is the result of the activity of the damaged intracellular substrate. The destruction is not a direct consequence of the variation of the content of nucleic acids in the nuclei. The regeneration of the irradiated tissues occurs from potentially viable cells and is limited first of all by the number of cells having lethal chromosome injuries and also by the reduced mitotic activity. Prophylactic defensive agents exert their action at the cellular level. Most agents protect by producing hypoxia in the radiosensitive tissues. Some sulphur-containing protective agents do not reduce the content of oxygen in the tissues.

Various changes in a mammalian organism arise as a result of exposure to ionizing radiation. It is evident, however, that only some injuries play an important role in the mechanism of the radiation reaction. At the present time radiation injury after exposure to doses as high as 10 to 15,000 r is considered to be determined first of all by the state of the haematopoietic and digestive systems. (This thesis is supported by numerous experiments on shielding and local irradiation which showed that peaks of mortality were related to damage to haematopoietic organs and to different parts of the digestive system (Maisin, *et al.*, 1957; Quastler, 1956; Quastler and Zucker, 1959; Rajevsky, 1956; Semenov and Fedorov, 1959).

The analysis of changes in these radiosensitive systems shows that the most characteristic feature of their reaction to irradiation is a mass destruction of cells which occurs soon after exposure. Destructive processes in some other organs (for example in the gonads) are of a local significance and have no effect on the survival of irradiated animals. The understanding of the mechanism of radiation injury and the elaboration of rational radiation protection require an investigation of

causes bringing about the death of cells and conditions for tissue regeneration.

Some questions concerning the causes of cell damage, tissue and cell repair and the mechanism of action of some protective agents are discussed in this paper.

## I. PROCESSES OF CELL INJURY

The death of irradiated cells that are able to undergo division is due to chromosome unbalance or to gross structural damage to the chromosomes which often stops the cells from completing mitosis.

1. Most of the irradiated cells of mouse cornea with bridges and acentric fragments were found to have died in the first generation. A complete elimination of such damaged cells takes place in the first-third generation; some cells die during mitosis (Shapiro and Konstantinova, 1959).

When irradiated cells do not enter mitosis, chromosome injuries are preserved in them during the whole interkinetic period. Thus about the same percentage (60 per cent) of liver cells with anaphase bridges and acentric fragments was observed in rats exposed to 500 r independent of the time between irradiation and hepatectomy (1 day, 2 or 4 months). The number of cells with chromosomal aberrations was only 25 per cent when partial hepatectomy was performed twice: 1 day and 1 month after total-body irradiation (Shapiro, 1959). Similar results were obtained on the liver of irradiated mice (Albert, 1958).

The data show that some chromosomal injuries, which become apparent during mitosis and lead to cell death, are preserved for a long time during interkinesis and have no visible effect on cellular viability. This mechanism of cell death, however, is not unique. Mass destruction of cells takes place in radiosensitive systems. This process develops within a few hours of exposure and, as will be shown below, it is not related to mitosis.

2. Mass destruction of cells proceeds only in directly irradiated parts of radiosensitive systems and does not occur in shielded parts.

This thesis may be illustrated by the results of experiments on local X-irradiation with 700 to 1,000 r of different portions of the haematopoietic system of mice. Cell destruction takes place only in the irradiated areas (Fig. 1a, b). The number of cells did not change significantly in the shielded areas. (Grayevsky, *et al.*, 1958; Barakina, 1959a).

3. The radiosensitivity of cells *in vivo* and *in vitro* is almost the same. Puck (1960) and Puck *et al.* (1957) have shown that various normal

and malignant human and animal cells when irradiated lost their ability to form macrocolonies after explantation and died, probably, because of chromosomal aberrations. The  $LD_{37}$  for these cells ranged from 50 r to 150 r.

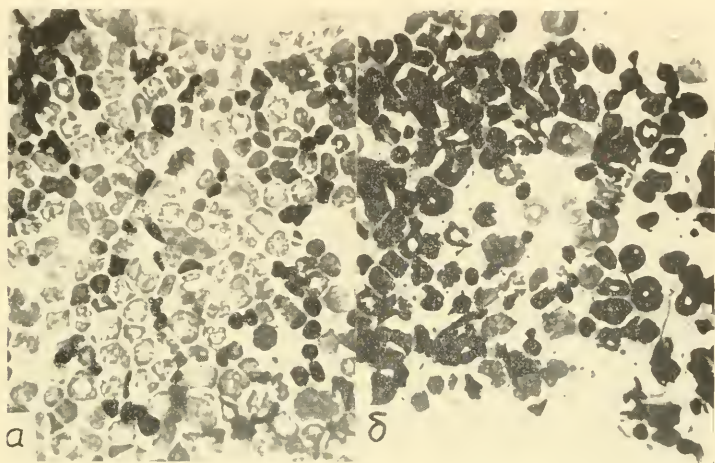


Fig. 1.—Mouse bone-marrow 6 hr after total-body X-irradiation with 700 r. (a) shielded portion; (b) irradiated portion.

Hematoxylin and eosin.  $\times 400$ .

Most mouse bone-marrow cells are known to undergo destruction after total-body irradiation with 700 r. At the same time a suspension of these cells *in vitro* did not undergo destruction when exposed to 700 to 1000 r. However, such cells lost their ability to restore haematopoiesis after injection into lethally-irradiated mice (Fig. 2) which may probably be explained by the death of these cells in the host organism. As a matter of fact cells irradiated *in vitro* and injected directly into the spleen of a normal mouse soon underwent destruction (Barakina, 1959b). McCulloch and Till (1960) injected lethally irradiated mice with isologous bone-marrow cells X-irradiated *in vitro* or *in vivo* with various doses. They found that the  $LD_{37}$  for these cells was 105 rad. The  $LD_{37}$  for mouse leukemia cells is equal to 160 r of  $\gamma$ -rays of  $^{60}\text{Co}$  (Hewitt and Wilson, 1959).

4. Cell destruction after irradiation does not take place in artificially isolated parts of the haematopoietic system.

No destruction of cells was observed: (a) in portions of spleen explanted in tissue culture or incubated *in vitro* after exposure and also in an irradiated suspension of bone-marrow cells (Barakina, 1959b;

Ivanitskaya, 1956); (b) in a removed spleen (or its portion) left in the abdominal cavity after exposure; (c) in a spleen whose nerve-vessel bundle was clamped after irradiation. Typical necrobiotic processes developed when the ligature was removed (Barakina, 1959b).



Fig. 2.—The average number of bone-marrow cells (b.m.c.) per field of vision (as a percentage of the normal number) in mice totally X-irradiated with 700 r.

1, irradiation only; 2, irradiation + intravenous injection of homologous b.m.c., X-irradiated with 700 r *in vitro*; 3, irradiation + intravenous injection of intact homologous b.m.c.

These data suggest that the death of irradiated cells is probably due to the loss by the injured, intracellular substrate of the ability to perform some function specific to it under normal conditions. If one can create conditions preventing the function of damaged substrate one can succeed in prolonging the life span of the irradiated cells.

5. The early mass death of haematopoietic cells and those in intestinal crypts is not related to mitosis or to chromosome rearrangements.

About 60 per cent of bone-marrow cells underwent necrosis after X-irradiation of the mouse with 700 r (Barakina, 1957, 1959c, and Fig. 3). Mitotic index in the mouse bone-marrow is equal to 1 per cent, and the duration of the resting stage is 4 to 6 days (Widner *et al.*, 1951). Taking into consideration that the exposure to 700 r sharply



decreases the rate of cell multiplication it seems evident that only a small number of cells have time to undergo division in 24 hr (the period when the early death is completed). Some differentiated cells (lymphocytes) and differentiating ones which had lost the ability to divide (neuroblasts in brain) also underwent destruction after irradiation (Trowell, 1952; Hicks, 1953).

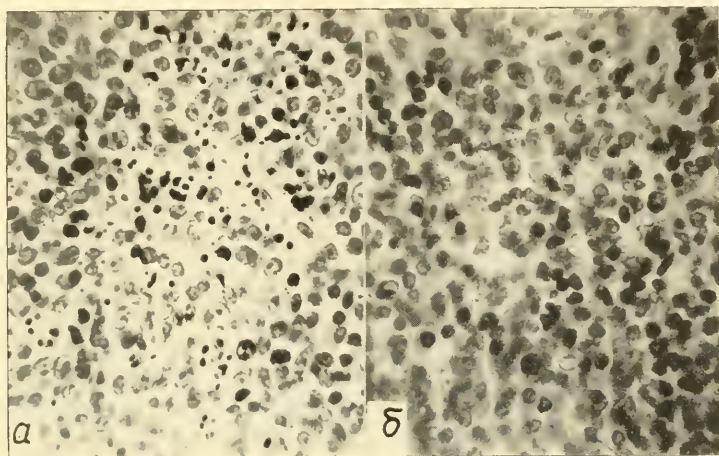


Fig. 3.—(a) Destruction of bone-marrow cells, X-irradiated with 700 r *in vitro* and injected into the spleen of an intact mouse; (b) absence of destruction of normal bone-marrow cells, injected into the spleen 4 hr after injection.

Hematoxyline and eosin.  $\times 600$ .

Early cell death is probably not the result of some radiation-induced chromosome aberrations (or breaks). There are a number of tissues whose cells do not undergo early death despite the fact that most of the irradiated cells have chromosome injuries. Thus the number of cells with anaphase bridges and acentric fragments in mouse cornea is about 90 per cent after exposure to 700 r and 60 per cent in rat liver, after treatment with 500 r of X-rays. Destruction of these cells prior to mitosis does not occur, however, even after irradiation with considerably higher doses (Shapiro, 1959; 1960).

Intensive processes of mitosis and differentiation are peculiar for most cells undergoing early death. In this case mitosis was shown not to bring about early cell death. Possibly it is the differentiation which realizes a latent radiation injury and causes cell destruction.

6. One of the main radiation effects is an inhibition of mitosis. Radiation-induced decrease in mitotic activity is related to nuclear injury.

This thesis may be proved by: (a) an almost equal delay of the first

cleavage after exposure of gametes (Henshaw and Frances, 1936; Shapiro *et al.*, 1960); (b) the absence of delay after irradiation of the anucleate half of the sea urchin egg and an inhibition of division after the exposure of the nucleated portion of the egg (Henshaw, 1938); (c) the absence of delay in the cleavage of the eggs (*Misgurnus fossilis* L.) X-irradiated during cleavage or at the early blastula stage when divisions proceed independently of nuclear structure preservation (Shapiro and Lander, 1960a; Shapiro *et al.*, 1960); (d) the absence of mitotic delay after irradiation at the middle blastula stage of the eggs if the nuclei were preliminarily strongly impaired by radiation. Exposure of normal embryos at this stage always induced temporary or constant (depending on the dose of radiation) delay of division (Shapiro *et al.*, 1960; Shapiro and Lander, 1960b).



Fig. 4.—The average number of bone-marrow cells (b.m.c.) per field of vision (as a percentage of the normal number) in mice totally X-irradiated with 700 r.

1, irradiation only; 2, irradiation + intravenous injection of isologous b.m.c.; 3, irradiation + intraperitoneal injection of isologous b.m.c.; 4, irradiation + intravenous injection of heterologous b.m.c.; 5, irradiation + intraperitoneal injection of heterologous b.m.c.

7. Destruction and changes of mitotic activity developing as a result of irradiation are not directly connected with changes in the content of nucleic acid derivatives.

The content of nucleotides and nucleosides in the nuclei of mouse bone-marrow cells was shown to decrease markedly in the very first

hours after X-irradiation. This decrease is a result of distant action as it is almost the same in the directly irradiated and in the shielded parts of the bone-marrow (Brodsky *et al.*, 1959). Taking into consideration that destructive processes and characteristic changes in the rate of multiplication occur mainly in directly-irradiated tissues it may be suggested that these changes are not a consequence of disturbances in nucleic acid synthesis.

Delay of division and cell destruction were found by some other authors to be not directly connected with the concentration and the synthesis of nucleic acid substances (Howard and Pele, 1953; Whitefield, 1959; Biagini *et al.*, 1958; Hjort, 1960).

## II. THE REPAIR OF TISSUE DAMAGE

The viability of an irradiated organism depends first of all on the rate of regeneration of its haematopoietic and digestive systems.

1. The repair of these systems is beyond any doubt due to the multiplication of either residual or injected viable cells. The following data support this concept: (a) preservation in an irradiated organism of intact portions of haematopoietic tissue (to a great extent independently of their volume) strongly accelerates the regeneration of haematopoietic organs depleted by irradiation (Grayevsky, *et al.*, 1958; Barakina, 1959a); (b) injection of an irradiated organism with intact bone-marrow cells has the same effect (Lorenz *et al.*, 1951; Nowell, *et al.*, 1956). Injection of disrupted or injured cells is without effect (Barakina, 1959b; Soška, 1958; Cole *et al.*, 1953). Other things being equal, cells of isologous bone-marrow exert a greater therapeutic effect than homo- and heterologous cells. Better results can be obtained after intravenous than after intraperitoneal injection (Van Bekkum and Vos, 1957; Barakina, 1960, Fig. 7); (d) Experiments in which cytological, cyto-chemical and immunological methods were used are the most convincing proof that the repopulation of haematopoietic organs in irradiated animals proceeds at the expense of dividing intact donor cells even if the latter belong to a species other than the recipient (Ford *et al.*, 1957; Vos *et al.*, 1956; Makinodan, 1956).

The role of humoral factors in the restoration of haematopoietic organs especially in some later period after exposure remains thus far obscure.

2. Chromosome aberrations and an inhibition of mitotic activity are the factors limiting the regeneration of irradiated tissues.

The more cells without lethal chromosome aberrations that are preserved after irradiation the more rapid will be the regeneration of

tissues, other things being equal. On the other hand, the higher the mitotic activity in irradiated tissues the more intensive the process of restoration. However, the main factor limiting regeneration ability after treatment is the quantity of cells with lethal chromosome rearrangements, since the non-viable offspring of these cells cannot provide for tissue repair despite their intensive multiplication.

Doses of 700 to 1000 r of X-rays seem to bring about the formation of lethal chromosome rearrangements in an overwhelming majority of cells of different tissues. For example (see Fig. 5) 90 to 95 per cent of

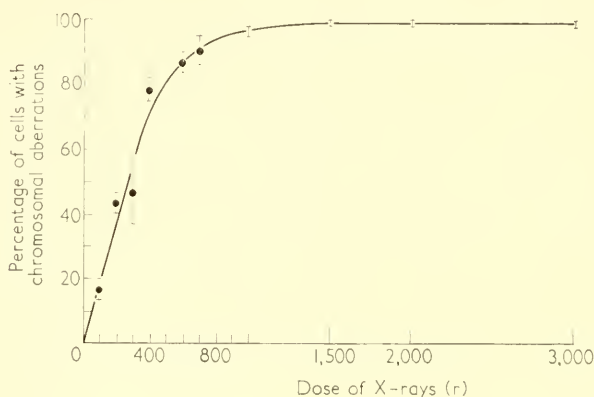


Fig. 5.—Dose-dependence of the percentage of cells with anaphase bridges and acentric fragments in the epithelium of mouse cornea.

the cells of mouse cornea were found to have anaphase bridges and acentric fragments after exposure to 700 to 1000 r. The mitotic index reached a rather high level after a period of complete inhibition of division even though higher doses of radiation were given Fig. 6 (Shapiro, 1960).

3. The repair of radiation injury at the cellular level may be a result of restitution or substitution of damaged structures and functions. The recovery of the ability of a cell to divide may be an example. Relatively low doses of radiation induce a temporary loss of ability to undergo mitosis followed by the return of this process to normal. Only partial recovery of mitotic ability occurs after irradiation with high doses. Without a special observation on a single cell it is difficult to be sure whether this lowering of the mitotic index is caused by the loss of ability to divide in a portion of cells or by a prolongation of interkinesis.

It was shown on *P. caudatum* cultured as a single cell that this unicellular organism recovered its ability to divide in a short time after irradiation. Changes in the tempo of division were the same after exposure of intact cells to 100,000 r as of those preliminarily irradiated with high doses (Grayevsky and Zinovieva, 1958).

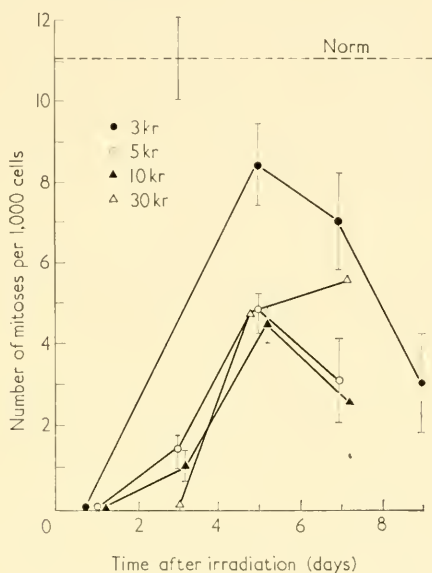


Fig. 6.—Mitotic index of the epithelium of mouse cornea after X-irradiation.

4. Realization of injury depends on the conditions of environment after irradiation. Manifestation of radiation damage is related to a function of the affected substrate.

The conditions preventing the destruction of irradiated bone-marrow cells were described above. It is possible to suppose that the repair of such an injured but not functioning substrate may take place in these particular conditions. The process of spontaneous recovery was found in gametes of *Drosophila* (Lüning, 1958) and in yeast (Korogodin, 1958). There are some data to show that the environment can promote development of the damage of such nonfunctioning substrates. For example, storage or germination of irradiated seeds in an atmosphere of oxygen brings about the realization of some potential chromosome breaks (Adams and Nilan, 1958; Caldecott *et al.*, 1957).

In some cases the injury to cells does significantly change after irradiation, in this connection accumulation of damage occurs after



repeated treatments corresponding to the total dose. This holds true for radiomutations (Kaufman, 1954) and, apparently, takes place in liver cells resting after irradiation. In this case the number of cells with aberrations (judging from the percentage of cells with anaphase bridges and fragments) did not change during interkinesis and the effects of repeated exposure were additive (Shapiro, 1959).

The importance of environmental conditions as a factor promoting or preventing the manifestation of radiation damage of cells has been shown also in experiments on *P. caudatum* (Litvinova, 1959a, b) and on *E. coli* (Hollaender, 1957). Changing the temperature of incubation (for paramecia) or the concentration of salts in the medium (for *E. coli*) significantly altered their reaction to radiation.

### III. SOME QUESTIONS OF RADIATION PROTECTION

1. The action of protective agents takes place at the cellular level. This conclusion is based on the following data:

(a) Local cooling of the rat skin (Evans *et al.*, 1942), clamping the tail (Howard-Flanders and Wright, 1957), limbs (Zhrebrenko *et al.*, 1959) or vessels of some internal organs (Weiss, 1959; Osborne and Solem, 1959; Martin, 1960) exert a distinct protective effect on corresponding organs and tissues.

(b) Application or injection of some substances has been shown to lower the radiation injury in the region of treatment (Forssberg, 1950; Kublitska'a, 1951; Fogh, 1960).

(c) Protective agents decrease the action of radiation on somatic (Bases, 1959) and tumour cells (Gray *et al.*, 1953; Conger, 1956; Tolkacheva, 1959) growing *in vitro* and on some plant cells (Lea, 1955; Giles and Riley, 1950; Thoday and Read, 1947, 1949), unicellular animals (Bacq *et al.*, 1952) and micro-organisms (Hollaender and Stapleton, 1953).

According to our data, the action of various radiation protective agents is postulated to be related to the local defence of cells in radio-sensitive systems. Cytological analysis of haematopoietic organs in mice irradiated with 700 to 1000 r in an atmosphere of carbon monoxide showed that (a) the process of cell destruction in the bone-marrow was significantly less: the number of cells preserved was about 1.5 to 2 times that in the unprotected control (Barakina, 1957, 1959c); (b) the regeneration of haematopoietic tissue proceeds much more rapidly than in the control (Barakina, 1957). The acceleration of the regeneration in this case is probably related to the preservation of a larger number of viable cells and to their more rapid multiplication judging from the

experiments on mouse cornea. Thus the number of cells having no bridges and fragments in the cornea of mice irradiated in an atmosphere of carbon monoxide was two times and the mitotic index 1.5 times that of the unprotected control (Shapiro and Konstantinova, 1959).

(2) The action of most protective agents is due to hypoxia of the tissues. Some S-containing substances (monothiois and thiourea derivatives) exert their action without causing hypoxia.

A decrease of  $O_2$ -tension during irradiation is known to weaken the radiation effect. The universality of this phenomenon led to the idea that the protective action of various agents could be explained by the hypoxia induced by them. However, until lately there were no direct experiments showing that protective substances change  $O_2$ -tension in tissues. That is why in 1957 we began experiments in which the influence of protective substances on the survival of  $\gamma$ -irradiated mice at different time intervals after drug-treatment was compared with the action of these agents on  $O_2$ -tension measured by a polarographic

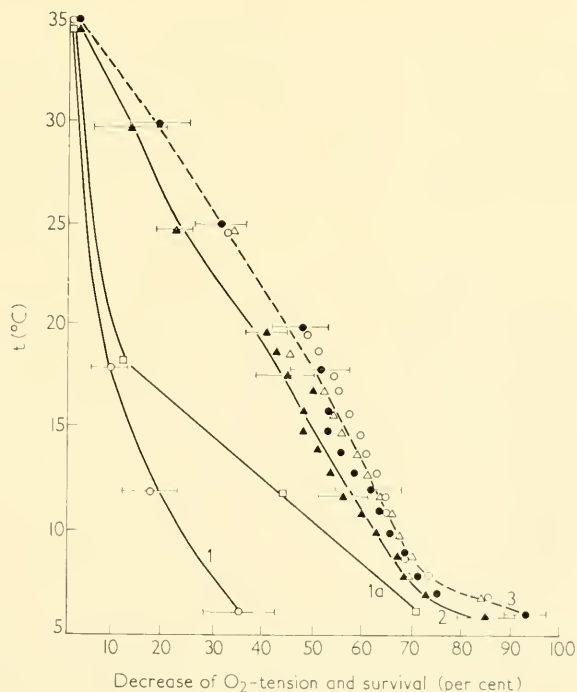


Fig. 7.—1, survival of mice exposed to 900 r of  $\gamma$ -rays of  $^{60}Co$  at different rectal temperatures. 1a, as 1 but taking into account the mice that died after cooling. 2,  $O_2$ -tension (percentage of the initial level) in the spleen ( $\blacktriangle$ ,  $\Delta$ , after cooling and rewarming, resp.). 3, the same in the liver ( $\bullet$ ,  $\circ$ , after cooling and rewarming, resp.)

method in some organs (Konstantinova and Grayevsky, 1960). These experiments should determine whether the protection is always related to hypoxia and whether all forms of hypoxia are capable of providing protection.

The following results were obtained in the experiments:

1. Some protective effect of hypothermia is observed for the first time at a rectal temperature of 18 to 20°C; this effect increases with temperature decrease down to 6 to 8°C and is accompanied by a diminution of O<sub>2</sub>-tension in the spleen and the liver by 15 per cent as compared to the initial level (Konstantinova, 1960, and Fig. 7).

2. Substances blocking the transport of oxygen by haemoglobin (CO, NaNO<sub>2</sub>) exert a distinct protective effect which depends on the

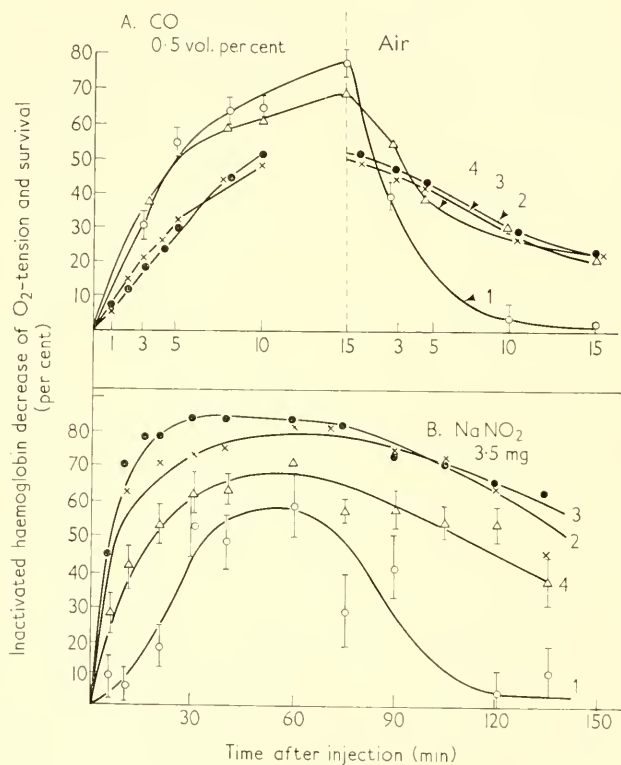


Fig. 8.(a)—1, dependence of a survival of mice  $\gamma$ -irradiated with 900 r of  $^{60}\text{Co}$  (600 r per minute) upon exposure to carbon monoxide (0.5 vol. per cent) followed by air. 2, O<sub>2</sub>-tension (percentage of the initial level) in the spleen. 3, in the liver and CO-Hb content in irradiated mice under the same conditions.

(b)—1, survival of mice  $\gamma$ -irradiated with 900 r at various time intervals after injection of NaNO<sub>2</sub>. 2, O<sub>2</sub>-tension in the spleen. 3, in the liver. 4, Mt-Hb content in non-irradiated mice at a corresponding time interval after injection.

amount of COHb formed and on the O<sub>2</sub>-tension in the tissues (Grayevsky and Konstantinova, 1958 and Fig. 8).

3. The maximum protective action of adrenaline, morphine and heroin corresponds to the period of maximal decrease of O<sub>2</sub>-tension in the liver and in the spleen (Fig. 9; Konstantinova and Grayevsky, 1960).

4. Dimercapto-compounds (BAL-intravenous, dimercaptopropionic acid) have been found to protect during the period of maximum decrease of O<sub>2</sub>-tension in tissues (Fig. 9; Grayevsky and Konstantinova, 1960a).

5. Strong reducing agents, ascorbic acid (40 mg per mouse), pyrogallol (5 mg), hyposulphite (60 mg) fail to bring about hypoxia of tissues and do not possess radioprotective action (Konstantinova and Grayevsky, 1960).

6. KCN (0.15 mg per mouse) while causing cytotoxic hypoxia only slightly decreased O<sub>2</sub>-tension in the tissues. We could not find a protective effect of KCN either on mice or on micro-organisms (Konstantinova and Grayevsky, 1960; Grayevsky and Konstantinova, 1957).

7. Monothiols and thiourea derivatives (cysteine, SH-glutathione, cystamine, cysteamine, 2-aminoethylisothiuronium BrHBr, 2-amino-5-isothiuronium methyl-thiazoline Br HBr) exert distinct protection without changing the level of O<sub>2</sub>-tension in tissues (Fig. 10; Grayevsky and Konstantinova, 1960b).

### CONCLUSIONS

Some questions concerning the processes of damage and recovery in irradiated organism are discussed. Death of a significant proportion of cells of haematopoietic organs and in intestinal crypts and inability of the tissues to regenerate quickly enough undoubtedly play an important role in the mammalian radiation syndrome.

Causes of the early mass destruction of cells in radiosensitive systems which develops soon after exposure have been insufficiently studied though this process is one of the most important consequences of irradiation. It has been shown that: (1) early mass destruction takes place only in directly irradiated tissues; (2) radiosensitivity of bone-marrow cells *in vivo* and *in vitro* does not differ significantly; (3) the early destruction is unlikely to be related to the process of division or to some radiation-induced chromosome damage; (4) early death occurs only in those cases where an affected substrate is put into operation. The specific function which makes radiation damage apparent may be assumed to be the process of differentiation.

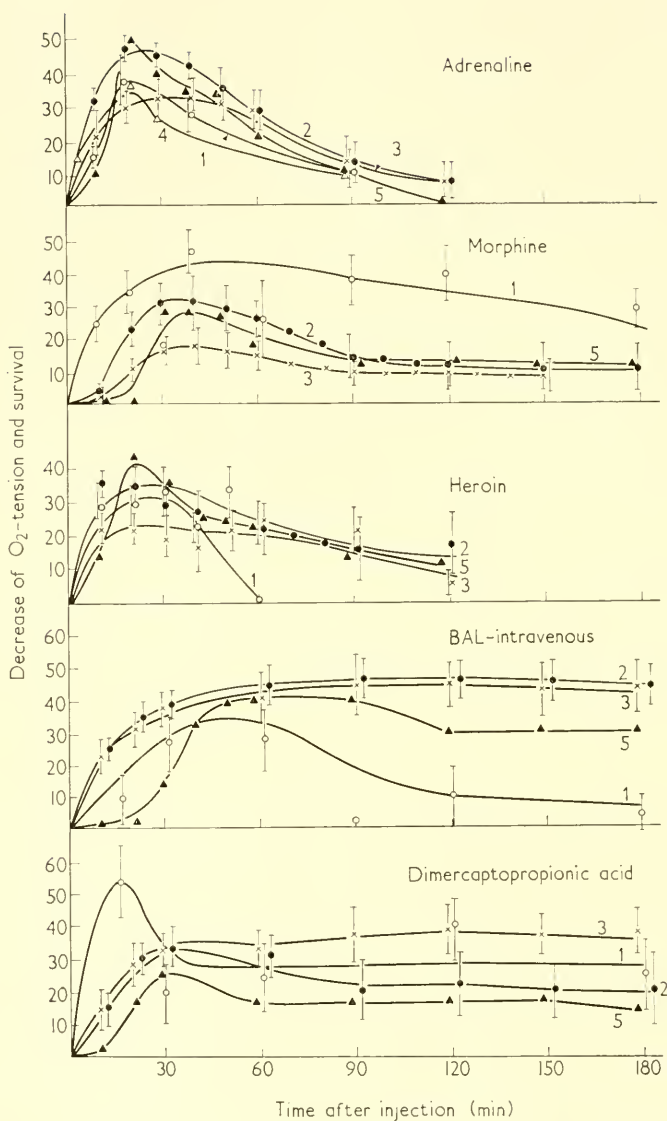


Fig. 9.—1, survival of mice  $\gamma$ -irradiated with 900 r at various time intervals after injection of a drug. 2, O<sub>2</sub>-tension in the spleen. 3, in the liver. 4, in muscle in non-irradiated mice at the same time after injection. 5, percentage of mice in which the decrease of O<sub>2</sub>-tension in the spleen was more than 50 per cent.



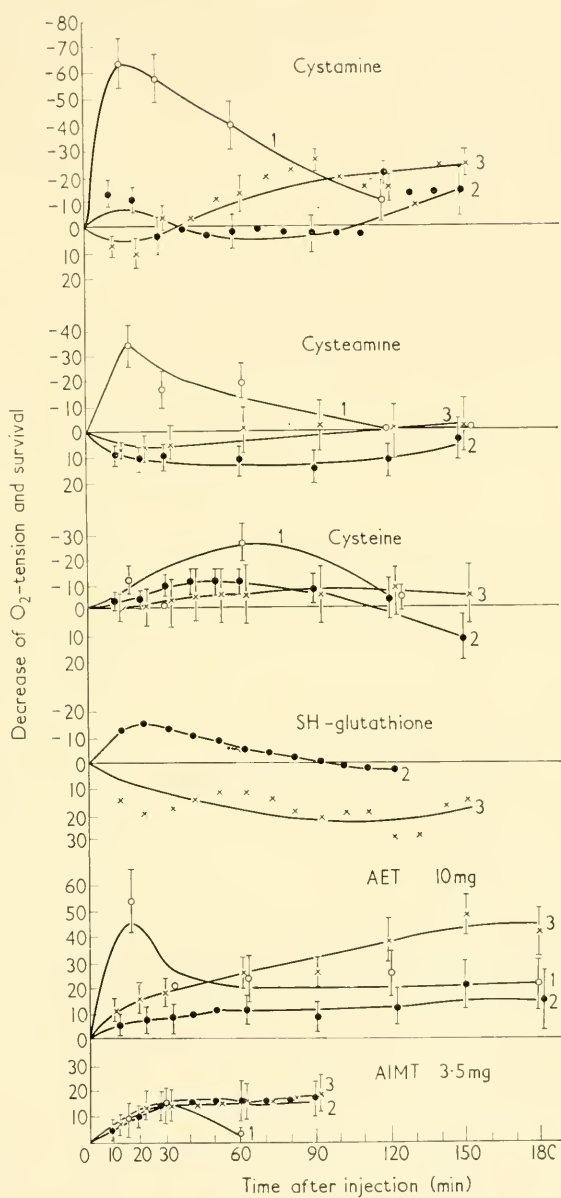


Fig. 10.—1, survival of mice  $\gamma$ -irradiated with 900 r at various time intervals after injection of a drug. 2, O<sub>2</sub>-tension in the spleen. 3, in the liver of non-irradiated mice at the same time interval after injection.

The death of cells remaining after early mass destruction is due to chromosome unbalance and to inability to complete division because of gross structural injuries to the chromosomes; the death of such cells is observed in generations F<sub>1</sub> to F<sub>3</sub>.

The main factor limiting regeneration of tissues exposed to 700 to 3,000 r has been shown to be the small number of residual viable cells since most cells have lethal chromosome aberrations. The number of such injured cells reaches 90 per cent after exposure to a minimum lethal dose (700 r of X-rays for mice).

The data presented show that the realization of radiation damage depends in some cases on environmental conditions. Delay of the development of injury may in some cases contribute to recovery. But even if the process of spontaneous repair does not play any significant role, latent damage may be influenced experimentally.

Reasons for the different radiosensitivity of haematopoietic and digestive systems are still unexplained. From the viewpoint of cellular radiobiology these differences may be due to: (1) a different number of cells affected by the early mass destruction; (2) a different number of cells with lethal chromosome damage; (3) a different rate of cell multiplication; (4) differences in the minimum number of cells necessary for the preservation of the viability of the irradiated animal. The answers to these questions will clear up some peculiarities of "bone-marrow" and "intestinal" death.

Thus, cellular changes underly the mammalian radiation syndrome. Radioprotective agents exert their influence on the organism by attenuating the action of radiation on the cells; the number of viable cells is thus increased and the recovery of mitotic activity proceeds more rapidly. As a result, the regeneration of damaged systems becomes more intensive.

A series of agents (hypothermia, neurotropic substances, substances linking to haemoglobin, dimercapto-compounds) have been shown by the direct measurements of O<sub>2</sub>-tension in tissues to protect by decreasing O<sub>2</sub>-tension in the tissues. The protective effect appears when O<sub>2</sub>-tension falls to 50 per cent of the initial level.

Protective action of the agents mentioned above is due to hypoxia in the cells of radiosensitive organs by accumulation of substances in cells, blocking up of O<sub>2</sub>-transport, inhibition of respiratory centres, spasm of vessels, etc. Among various forms of hypoxia only the histotoxic one exerts no protective action because it does not significantly change the O<sub>2</sub>-tension in the tissues. The radioprotective action of hypothermia in homoiothermal animals is related to hypoxia arising, apparently, as a result of the fact that tissue respiration is inhibited more

strongly than the transport of oxygen to tissues. It may be suggested that this event does not take place in heterothermous animals irradiated under lowered body-temperature during hibernation; no hypoxia of tissues is brought about under these conditions and no protection occurs.

A number of protective agents (monothiols and thiourea derivatives) possess a strong protective action but do not cause hypoxia. This fact does not exclude the possibility that the mechanism of action of these substances is also associated with the oxygen effect but this question requires special investigations.

It should be stressed that the efficiency of all the substances tested varies strongly with time. It may be just this fact which accounts for the different data found in the literature. In order properly to evaluate an agent, its efficiency should be tested at different time intervals after injection.

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## DISCUSSION

SHABADASH: You know about experiments performed by Drs. Pap and co-workers, Nemenov, Gorizontov and Grafov, as well as by others, which have

shown that local irradiation of the diencephalon, i.e. hypothalamic region, with small doses brings about typical "radiation sickness" in the bone-marrow.

I would like to know how you excluded the influence of the hypothalamus on haematopoiesis in the case of total body irradiation and whether there have been qualitative and quantitative differences in the cell degeneration process following local irradiation of the limb.

GRAYEVSKY: As I have said already Dr. Barakina has found that cell destruction occurs only in directly irradiated areas; there was no destruction in the screened areas. We failed to establish qualitative and quantitative differences in the cell destruction process occurring in the haematopoietic organs following total body and local irradiation. During the repair stage the difference was that following local irradiation regeneration proceeded more rapidly. This is accounted for by the preservation in the body of intact haematopoietic elements.

TOBIAS: I did not quite understand what protective substance was used in the case of catalase. Can you not give more detailed information about the degree of the protective action of this substance when aqueous solutions of catalase were irradiated?

GRAYEVSKY: In the experiments to which you refer we have studied the radiation effects on the catalase both in solution and in the unicellular organism (*P. caudatum*). Irradiation of the catalase solution with 10,000 r brought about destruction of this enzyme, whereas in the cell the enzyme was not inactivated even when doses of the order of 100,000 r were used.

ALEXANDER: Have you any observations on a spontaneous restoration of the lesions in the liver cells?

Have you data that would make it possible to conclude how much time is required for the repair of the destroyed chromosomes in liver cells?

GRAYEVSKY: Dr. Shapiro studied this problem on rat liver cells. He has shown that the number of cells with anaphase bridges and acentric chromosome fragments remains the same irrespective of the time after exposure (1, 2 or 4 months) when the hepatectomy was performed. Hence it follows that in resting cells there is a persistent chromosome damage induced by radiation.

He has also shown with mouse corneal epithelium that the cells with chromosome damage perish in the first to third generation.





# STUDIES ON ENZYMES AND YEAST CELLS WITH ACCELERATED HEAVY IONS

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## SUMMARY

The action of heavy accelerated ions, with short-range but very high linear energy transfer, on enzyme molecules, such as trypsin, in the dry state can be modified by the presence of the protective substances, dextran, lactose and ribose. The inactivation cross-sections obtained are discussed in the light of current theories.

Heavy ions produce mutations in irradiated yeast cells and inhibition of cell division and colony formation. The effects produced depend upon the post-irradiation physical environment and the physiological state of the cells.

## INTRODUCTION

For the past several years my collaborators and I have been interested in the effects of heavy accelerated ions on living cells and molecules of biological importance. Over the past three years, using the Berkeley heavy ion linear accelerator (HILAC) machine strong pulsed beams of protons, alpha-particles, Boron-10, Carbon-12, Nitrogen-14, Oxygen-16, Neon-20 and Argon were available (Born *et al.*, 1959). These particles have short range but very heavy linear energy transfer (LET). Figure 1 shows that irradiations of cells and thin layers of molecules is possible by the track segment method up to LET of  $10^{10}$  eV/g cm<sup>-2</sup>. The radiobiological use of such particles is essential primarily because they help us to understand the primary action of radiation and the mechanisms of molecular energy transfer. The presence of heavy ions in the primary cosmic radiation in outer space makes their study even more interesting. If man should fly in space he will be exposed to some rather penetrating heavy particles. It is further assumed that heavy cosmic ray primaries bombard the surfaces of planets where generation of organic matter might still be in process. On planets with low atmospheric pressure the heavy ions penetrate to the surface so that life, if it exists must continually go on in their presence.

† On leave of absence from the Norwegian State Cancer Research Laboratory.

Initial work was carried out seven years ago by Birge and Sayeg (1959) and Birge *et al.* (1959). Recently Brustad (1960), Fluke *et al.* (1961), Manney *et al.* (1960) and Mortimer and Brustad (1960) have

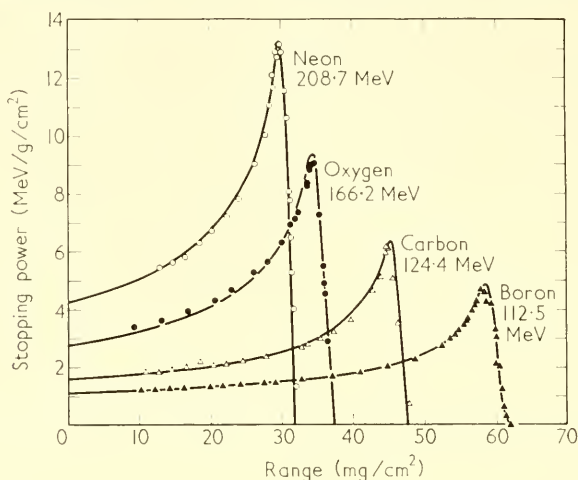


Fig. 1.—Bragg ionization curves for various accelerated heavy ions.

been engaged in heavy ion studies at Berkeley and similar work is being carried on at Yale University (Hutchinson, 1960).

We already know a great deal about the sensitivity of the cell nucleus to the effects of radiation: but we are not yet certain what kind of molecule is the critical species affected and how ionization and excitation energy travels within biological material. We suspect that there are chain reactions leading to cell death, yet we have not been able to define these precisely. Until recently it was believed that in the dry state, macromolecules must be affected directly by radiation whereas in aqueous systems the existence of indirect mechanisms have been repeatedly demonstrated. The purpose of this investigation is to show that even in dry molecules there is a considerable indirect radiation effect of sufficient magnitude that target theory type calculations may lead to misleading answers. Further we wish to add to the evidence that in aqueous systems even with the heaviest ionizing particles there is still a considerable part of the radiation effect mediated in water.

#### IRRADIATION OF ENZYME MOLECULES IN THE DRY STATE

The technique of mounting dehydrated samples of enzymes, irradiating and assaying them has already been described (Fluke *et al.*, 1961).

Results obtained on lysozyme, trypsin and deoxyribonuclease by Brustad are briefly reported here. When a sample of  $N_0$  enzyme molecules was irradiated by a dose of  $f$  particles per  $\text{cm}^2$   $N$  enzyme molecules remained which showed activity according to the well known relationship

$$N = N_0 e^{-\sigma f}$$

where  $\sigma$  is the interaction cross-section. Each of the radiated particles gave this sort of relationship with each of the enzymes studied, but with cross-sections different for each of the LET values at which irradiation of the molecule was carried out. According to a treatment by Pollard (1953) inactivation of the enzymes usually depends on producing a primary ionization within some definite part of the enzyme molecule; the cross-section is related to the number of primary ionizations per unit length  $k$  and the mean thickness of the enzyme molecule  $l$  by

$$\sigma = \sigma_0 (1 - e^{-kt})$$

Here  $\sigma_0$  is the geometric cross-section of the molecule or of its sensitive part. It may be obtained by radiating the molecule with very heavily ionizing rays: at high ion density the cross-section should become independent of LET. The data obtained in our laboratory do not agree with this model without further modification. Experimentally one does not obtain a plateau of cross-section with increasing LET. At the highest measured cross-section the values obtained are for lysozyme about 2.5 times the expected value, for trypsin 2.2 times and for deoxyribonuclease 1.27 times what one would deduce from molecular weight and other data known from independent sources. Although Pollard and Barrett (1959) and Fluke *et al.* (1961) attempted to work out methods whereby one would take into effect the  $\delta$ -rays produced by the primary particles and in effect increase the cross-section for enzyme inactivation. We believe that the modifying effect of  $\delta$ -rays are too small to explain completely the large cross-sections reported. While there can be no doubt that  $\delta$ -rays and their effects exist, some experiments were undertaken to see how neighbouring molecules and other environmental conditions might affect inactivation cross-section. It is possible to design experiments which may elucidate the role  $\delta$ -rays play in the inactivation cross-section. The oxygen effect provides one approach. It is known since the work of Alexander (1957) that oxygen can modify radiation injury due to lightly ionizing radiations (X-rays or electrons) in dry macromolecules. In fact trypsin exhibits an

increased radiosensitivity to oxygen by a factor close to two when radiated by 50 kV X-rays. One would expect a similar oxygen effect due to the more energetic  $\delta$ -rays in the track of a heavy ionizing particle whereas the densely ionizing core of the track may not exhibit an oxygen effect. For trypsin, carbon irradiation and exposure to even heavier ions does not show an oxygen effect at one atmosphere pressure within experimental error and we can safely say that the oxygen effect is less than 20 per cent of the oxygen effect with X-rays. Thus it appears that heavy ions exert most of their effect in the densely ionizing central portion of their track. Another test is to compare the effect of two different accelerated particles which have different atomic number but both of which have the same linear energy transfer. Since the two kinds of particles achieve the same LET by travelling at different velocities, the  $\delta$ -ray distributions will be somewhat different: the maximum velocity of  $\delta$ -ray electrons is approximately the same as the velocity of the primary particles. Comparison of data taken with neon and oxygen or carbon and boron particles has so far failed to disclose a definite  $\delta$ -ray effect.

The remaining explanation for the large cross-section is the assumption that the inactivated enzyme molecules do not all receive an ionizing particle passing directly through them, but sometimes they can become inactivated by interaction with neighbouring radiated molecules, be this chemical interaction, some sort of physical effect or excitation energy transfer. Norman and Ginoza (1958) indicated the existence of radiation protection of catalase by glutathione in the dry state, Hutchinson showed some years ago that the nature of aggregation of dry bovine serum albumin can alter its radiation sensitivity. Braams

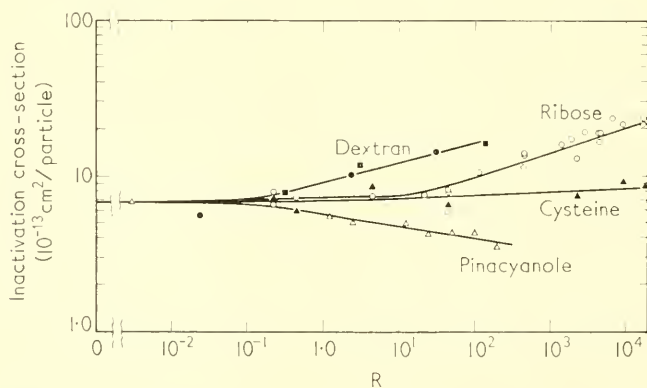


Fig. 2.—Modification of the radiation sensitivity of trypsin in the dry state by dextran, ribose, cysteine and pinacyanole. The data were obtained for accelerated carbon ions.



(1960) and Braams *et al.* (1958) found variations of radiosensitivity of dry enzymes when mixed with some inert materials. Figure 2 presents data showing that dextran and ribose increase the cross-section of trypsin, whereas pinaeyanole protects and cysteine leaves the cross-section unaltered. It is of interest to note that the protection increases as the concentration of the modifier is increased and that a total of four-fold variation in  $\sigma$  has been achieved. The protection afforded by lactose is shown in Fig. 3, and we may note that cysteine can reverse

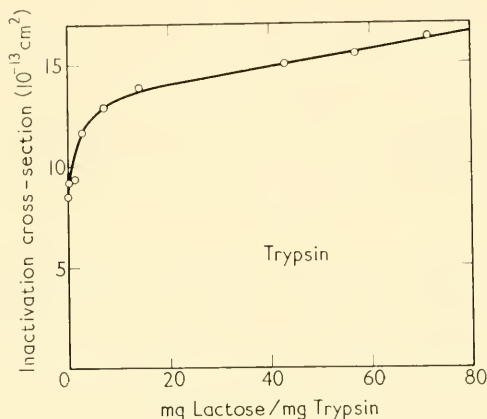


Fig. 3.—Increase of cross-section of dry trypsin by the addition of lactose.

lactose protection. In looking at the quantitative aspect of protection we may note that pinaeyanole and dextran, both small molecules already exert an effect at concentrations where there are insufficient modifier molecules present to cover the entire surface of the trypsin molecules. On the other hand in lactose and ribose enhancement of the radiation effect the cross-section is still increasing after the modifier molecules have covered the entire surface of the trypsin molecules. It would thus appear that in some instances an increase of cross-section is caused by modifier molecules which are as far as  $30 \text{ \AA}$  away from the enzyme molecules affected. Interpretations are made more difficult by the circumstance that the enzyme molecules are always dissolved in an aqueous system for assay and one does not know at which stage of the process a protective or enhancing effect occurs. We have also observed an effect of temperature on inactivation cross-sections, as did Hutchinson (1960). Further the pH of the solution from which the enzyme is dried has an effect. Results such as these make it particularly difficult to estimate how much of the radiation effects on enzymes in living cells

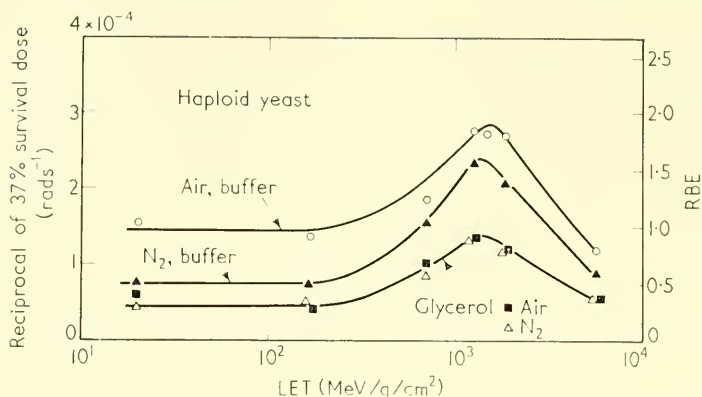


Fig. 4.—Relative biological effectiveness as function of linear energy transfer for haploid yeast cells in buffer and in glycerol, exposed to air or to nitrogen atmosphere. Note the protective effect of glycerol over the entire range of LET values.

in aqueous milieu is due to direct hit and how much is due to indirect mechanisms mediated by water, particularly if one realizes that in a living cell some of the enzyme may be in a specially protected inactive state; e.g. trypsin may be present as trypsinogen.

#### EXPERIMENTS WITH YEAST CELLS

Interpretation of dose-effect relationships is becoming exceedingly difficult when we realize that the effects can result either from direct effects on macromolecules, from indirect effects on the aqueous cell medium and also from macromolecular interactions following radiation in the denser regions of the cells, e.g. in nucleus or mitochondria. Some years ago Zirkle and Tobias (1953) proposed the "migration" model for explanation of radiation survival relationships obtained on cells of different ploidies. The model allowed reconciliation of indirect and direct action mechanisms. The effects have also been studied with low and high LET radiations. The cross-section of the resting wet haploid cell in air was found to be constant for the heaviest particles, above LET of  $10^9$  eV cm<sup>2</sup>/g and its value is about  $10^{-8}$  cm<sup>2</sup>. Experimentally it still has not been proved whether or not the radiation action has an intermediate component at high LET. We have attempted to determine if the aqueous medium of the nucleus is able to modify the heavy ion effects. Our initial results with X-rays agree with data presented by Gray at this conference. Wood has already shown that a change in phase state modifies the radiosensitivity of the haploid yeast cell to X-rays and that the increased radioresistance is about of the same

magnitude as one obtains by dehydrating the cells at room temperature by the use of glycerol. For X-rays it was also established that the yeast cells exhibited an oxygen effect (Birge and Tobias, 1954). Thus the current experiments performed by Manney *et al.* (1960) were directed at combined effects of glycerol-induced dehydration and the effect of gaseous environments. With X-rays 6 M glycerol in water caused a dose reduction factor of about 2.6 in aerated suspensions of cells. The cells were exposed to the various heavy particles on the surface of a millipore filter; survival scoring was made on the basis of colony counts from single cell isolates plated on full nutrient agar medium. The RBE has a peak for irradiation in air, in buffer with nitrogen or in glycerol. Glycerol protects the cells with respect to buffer with an approximately constant dose increase factor of 1.7 for all LET values compared to the nitrogen-buffer system and a factor of about 2.5 for the oxygen-buffer system, which decreases to about 2.0 for the highest LET tested. The RBE values are plotted in Fig. 4.

While haploid cells have exponential survival curves for all LET values, the shape of the diploid survival curves is modified at high LET values in a way somewhat similar to those described by Barendsen for human kidney cells. Correspondingly the RBE for high LET is about 3.4 for diploid cells. There appears to be more dominant lethal damage from heavy ionization. Whether this is due to chromosome breaks that fail to recombine, or a higher probability for the chromosomes to suffer multiple breaks from passage of a single heavy particle, we do not know at the present time.

Preliminary data obtained in our laboratory on the yield of mutations at specific loci as function of the LET indicates that some loci have a similar RBE to that of the inhibition of colony formation, others have a decreasing RBE at high values of LET (Mortimer and Brustad, 1960).

The mean dose necessary to kill a haploid yeast cell is, at heavy ionization, where the cross-section plateau corresponds to a cross-section of the approximate size of the nucleus of the yeast cells. Thus it appears, that, as in the case of lightly ionizing particles, radiation injury of heavy particles is particularly hazardous if interaction with the cell nucleus is involved.

These results, make one speculate as to the nature of heavy ion effects in the aqueous medium. Glycerol is not only an antidote for the oxygen effect but it also diminishes the effect in nitrogen. We might surmise that irradiated glycerol scavenges not only OH radicals, but oxygen as well. Or it may in some other, as yet unknown way, interfere with the mediating action of water molecules.

SURVEY OF RADIATION EFFECTS ON CELL DIVISION INHIBITION  
ON *S. CEREVISIAE*

Now I propose to describe briefly to you the present state of experiments and theories concerning radiation effects on the yeast cells, *Saccharomyces cerevisiae*. There are 10 strains of interest, ranging from haploid to hexaploid. Due to work by Latarjet and Ephrussi, Zirkle, Mortimer and myself, we now have the following general picture of the radiobiological events (See Zirkle and Tobias, 1953; Mortimer and Tobias, 1953; Mortimer, 1955; Birge and Tobias, 1954; Tobias *et al.*, 1959).

Inhibition of colony formation occurs by one of two major pathways: recessive or dominant lethal mutation.

In a diploid cell a homozygous pair of chromosome defects will cause inhibition of colony formation, whereas heterozygous, unpaired defects will not. This mode of effect can be directly demonstrated by sporulating the radiated diploid cells and discovering haploid spores with lethals in them.

The dominant lethal defect of the chromosomes is, by definition, one where a heterozygous lethal in one of a pair of chromosomes only, leads to inhibition of cell division. This effect is demonstrated by mating an irradiated haploid with an unirradiated one and demonstrating that some cases the diploid zygote is not viable.

The survival curves in presence of environmental modifiers of haploid and higher ploidy cells exposed to X-rays can be mathematically accounted for by the above mentioned two models for radiation effect. However, it was found that the post-irradiation physical environment of the cells and their physiological state modify the survival function. The major findings to be accounted for are:

1. Budding haploid cells show a tenfold increase in radioresistance and multi-hit survival curves.
2. Post-irradiation temperature treatment and changes in the composition of the nutrient medium modify the diploid macro-colony survival but not that of haploid cells (Korogodin *et al.*, 1959; see also presentation by Tarusov at the present conference).
3. Phenotypic appearance of mutations and production of lethals continue for at least a few generations following irradiation. In this period recovery from at least some of the genetic effects occurs.

In attempting to give a model for radiation action we should also discuss the types of genetic change that occur in irradiated yeast cells. The following major classes of genetic change have been observed:

- a. Deletions and productions of auxotrophic mutants; these are frequently recessive;



- b. Formation of chromosome bridges leading to dominant lethality.
- c. Increased occurrence of somatic cross-over. This interesting process will make a heterozygous genetic defect homozygous. When recessive lethal damage occurs, the cross-over process can result in a homozygous lethal daughter and another daughter cell which is free from genetic damage. Observation of this delayed genetic recovery process can be made in genetic loci, which lead to deficiencies causing colour in the affected cells. Adenine deficiency and galactose requirement can lead to sectorized colonies of this type.
- d. "Point mutations". These occur at loci well defined on the genetic linkage maps. These occur more frequently after ultraviolet, but X- and  $\alpha$ -rays can also produce them. They are normally spontaneously reversible. Many different point mutations are possible at the same locus.
- e. A most interesting genetic process is one first found with u.v. radiations by Roman. Here a homozygous but heteroallelic diploid cell is tested, having an auxotroph requirement. Radiation will allow the cell to become independent of the nutritional requirement. The explanation is either genetic recombination or "copy choice" replicating mechanism.
- f. X-rays and particularly u.v. can cause the loss from the cell of genetically controlled cytoplasmic particles, such as the cytoplasmic mutant of Ephrussi. This can occur either as a nuclear effect or as direct result of cytoplasmic absorption of radiation.

While each of the above classes of genetic damage forms the object of special studies, some very major problems still await solution. We do not know how initial molecular damage from a single ionizing particle is amplified to macroscopically observable or even lethal damage. It would be important to discover whether recessive and dominant effects originate from the same kind of initial lesion, with the difference in outcome being merely an environment-dependent probability factor, or whether the different end results stem from different initial lesions. It becomes more and more apparent that a cell, that has received near lethal radiation also has many sublethal lesions, which can modify its genetic and physiological characteristics. Lethality depends on environmental metabolic and physiological events in the period following radiation exposure.

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#### DISCUSSION

ALEXANDER: Why does the ploidy affect radiosensitivity differently for yeasts than for plants?

TOBIAS: With the ploidy increases the effect of the recessive lethals goes down, whereas that of the dominant ones increases. The outcome is determined by the interaction of these two processes. There is another yeast line, where the ploidy effect is different.

POLLARD: It is very important to consider the effect of  $\delta$ -irradiation by heavy ions, using Bohr's formula, as it was used by Lee and others. This was done for  $\beta$ -galactosidase by Hutchinson and Dolphin. They have shown that only one inactivation value may be used to explain the results obtained while using  $\delta$ -rays as well as irradiation by very heavy ions. The work with trypsin recently carried out in Yale, could also be interpreted from this standpoint.

As for the oxygen effect, since the quantity of  $\delta$ -rays varies in inverse ratio to the square of the energy  $\delta$ -ray intensity will be higher for lower energy radiations, showing very small oxygen effect. So according to these two considerations, the results of these experiments do not require in themselves assumptions about any additional effects. For other experimental data this may be the case, but to account for the results presented here there was no need for such effects.

TOBIAS: I agree with Dr. Pollard. Of course,  $\delta$ -rays should be studied carefully. I may only repeat the thesis, that we cannot explain all the effects observed by this phenomenon alone.

MARCOVICH: It is very interesting that when heavy particles were used, a protective effect was found and oxygen did not exert any influence.

Did you study the influence of glycerol on other organisms beside yeast?

TOBIAS: We have not used glycerol for other organisms. We are continuing this work; it has proven to be very complex.

MOUTON: I would like to ask about the effect of pH.

TOBIAS: This is also a very difficult question, since water itself changes the process.

ARDASHNIKOV: Have I understood you correctly that the addition of the protective agent in some cases brings about an increase of the sensitive volume? If this is so, then how should it be understood, since addition of the protective agent should increase the lethal dose, while increase of the dose must always produce a decrease of the sensitive volume, whatever be the modification of the basic formula for the calculation of the sensitive volume.

TOBIAS: In Fig. 1, I showed only the cross-section; the effective dose was not given. When the cross-section increases, it means that the same effect may be produced by a smaller dose.

GRAY: I congratulate Dr. Tobias on his excellent work on the effects of highly ionizing heavy particles.

I would like to make a remark connecting with Dr. Marcovich's statement. Observations made by Dr. Dewey on bacteria *Serratia marcescens*, to which I have already referred in this symposium, are in good agreement with Dr. Tobias' data with regard to the glycerol effect on damage caused by particles with low linear energy transfer. We observed a strong protective effect by glycerol (about two-fold) even if the bacteria were under strictly anaerobic conditions during irradiation. Since, however, aerobic cells are protected by glycerol to about the same degree when equilibrated either in 100 per cent oxygen or in 1 per cent oxygen, we think that the mechanism of protection is not connected with competition between glycerol and oxygen for the radicals.



# THE RHYTHM OF OXIDATIVE PROCESSES AND ITS DISTURBANCE UNDER THE ACTION OF RADIATION

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Vast experimental material has been accumulated by now on the principles of biological action of radiation, and promising theoretical generalizations have been made. Despite this, any attempt to approach this problem from new aspects should be sought for and used.

The present Symposium which focuses attention on the discussion of the current state of this problem, prompts us to put forward some speculations which could bring about a fruitful discussion.

It may sound paradoxical, but it seems that the development of radiobiology in its attempt to reveal chemical and physico-chemical mechanisms of radiation damage sometimes outraces or, at any rate, tries to outrace the general biological level of investigations. That is why in the search for new pathways and viewpoints, in the analysis of the physico-chemical bases of primary and secondary responses to the action of radiation, one should proceed from a thorough analysis of the present-day state of the problem of physico-chemical bases of life phenomena *sensu latu*.

Indeed, when one speaks of the extraordinary complexity of primary and initial chemical and physico-chemical processes in cells under the action of radiation, as is often done in the literature, this thesis should really be rephrased in the opposite form. It is the normal, strictly regulated course of life phenomena and the physico-chemical apparatus that ensures this proceeding which are more complicated and puzzling. As to the complexity of radiobiological phenomena, they are complicated inasmuch as they represent, figuratively speaking, a breakdown of a complex mechanism.

It became a tradition underlied by many years of hard work by biologists to regard life phenomena either from the viewpoint of morphology, or from that of metabolic processes, i.e. biochemistry. Until recently, the morphological aspect was moving to the background, having a flavour of conservatism, and gave way to the swift rise of biochemical aspects. Lately, however, the submicroscopical cell region,

with the introduction of electron microscopy, with the development of new, diverse, chemical and optical variants of histochemistry, has endowed morphology with a new meaning. Thus the science of the structure became confluent with that of the chemistry of living substrate.

This is the fundamental feature of the contemporary scene in the solution of biological problems.

Molecular, supermolecular and cellular organization are related to and, moreover, make possible the realization of metabolic processes which provide not only the self-maintenance of the cell but the processes of its development and self-reproduction as well.

The problem of the correlation between form and structure, posed by biologists as the central link of theoretical approaches to life phenomena, becomes nowadays, in accordance with all that has just been said more concrete from the physico-chemical and chemical aspects and can be re-analysed at a new level.

Thus, it seems that the central problem is that of the ability of cellular processes to stable self-regulation and, by analogy with modern technical terminology, to self-adjustment of this self-regulation to the most advantageous regime of work. Here not only the provision of metabolic processes in the spatial organization of cell structures but also the role and participation of these structures in the processes of self-regulation seem to be of fundamental significance. This sphere, which concerns the level of the very cell processes may be provisionally designated "microcybernetics."

From this viewpoint the problem of the physico-chemical bases of a destruction of the processes of cell regulation is a very important one when one considers the action of radiation on cells.

In principle this way of formulating the problem goes without saying and hardly deserves general and purely abstract discussion. We should like, however, if not to argue this viewpoint, to illustrate it at least, with some new facts, which can become the point of a fruitful discussion.

This way of setting out the problem arose for the first time in our laboratory some years ago when unexpected results were obtained on solving quite other problems. We were concerned with disturbances of haemodynamics after an acute action of radiation, especially clearly revealed on such experimental animals as rabbits.

In order to determine how these disturbances affect the oxygen balance of the tissues, Dr. Snezhko, in our laboratory, succeeded in applying a modification of a polarographic method to the continuous recording of the oxygen level in tissues of the irradiated animal.



As is well known, this procedure was applied for the first time by Davies and Brink (1942) for the direct measurement of the free oxygen tension in the tissues of a living animal. These workers developed various kinds of electrodes. We used the simplest modification consisting of the insertion of an open platinum needle. Without making it possible to determine the absolute oxygen content, this modification allowed us to demonstrate and to follow simply the relative changes in free oxygen content in tissues at the expense of the component determined by diffusion current which, in its turn, was determined by the concentration of free oxygen. These papers are published (Snezhko, 1956, 1957) and I shall not dwell on them any longer.

It was just this open platinum electrode which allowed us to realize the procedure of long term continuous recording of relative changes in free oxygen content. At the same time an accessory procedure, called "oxygen test" turned out to be a specified test and simultaneously the check of the behaviour of oxygen itself in tissues. For this experimental animals are exposed to a short inhalation of pure oxygen in strictly controlled doses. This leads to a short term enrichment of tissues in oxygen which is well registered polarographically. The level of this enrichment, as well as "resolving" the curve of the excessive oxygen concentration after the cessation of inhalation was found to be specific and served as a quantitative index not only of haemodynamic conditions but of the rate of oxygen utilization by the tissue as well.

In other words, with an increase of oxygen concentration in the air inhaled by the animal, the current (which is directly correlated with the concentration of tissue oxygen) increases for the time of inhalation. After the influx of this air ceases the current diminishes to the initial value. An increase takes place some 2 to 5 sec. after the start of the oxygen flow. Some seconds after the cessation of this flow the current still continues to increase. The current registered falls to its initial value 1.5 to 2 minutes after the arrest of the flow.

The advantage of this method is that the value for the accessory current designed as  $\Delta\tau$  in pure form is determined by the excessive oxygen concentration in the tissue, while, when applying the open electrode, the main current is partially related to side-phenomena.

Apart from this, the utilization curve of the excess amount of oxygen is, as was mentioned above, a good index of the functional state of the tissue.

In the experiments on rabbits, the behaviour of free oxygen in the tissues of the central nervous system was the first to be studied. For this several electrodes were inserted in different parts of the system. In our opinion, this was the start of the topographical study of processes

related to oxygen utilization. Various parts of the central nervous system were found to behave in an individual manner.

The first thing noticed when the experimental animal was irradiated either totally or locally to its head, was a picture just the opposite of the expected one. Despite a profound disturbance of the haemodynamics which began in the first hours after total irradiation, no impoverishment of the brain tissue in pure oxygen was found. This could be convincingly demonstrated with the use of the "oxygen test" which showed a distinct decrease in the ability of the tissue to utilize an extra supply of oxygen (Fig. 1).

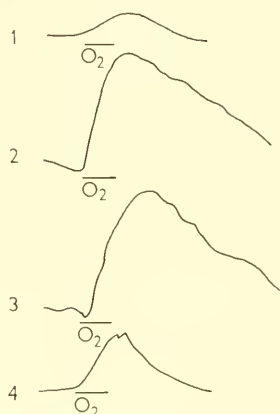


Fig. 1.—Change  $\Delta\tau$  in the result of X-ray irradiation.

1, before irradiation;

2, 1 hr after irradiation;

3, 5 hr after irradiation;

4, 7 hr after irradiation.

Thus, this approach to the investigation shifted the centre of attention from the observation of the after-effects of haemodynamic disturbances, as was expected earlier, to the observation of disturbances in tissue respiration.

It is evident that this method has important advantages over two procedures widely applied until recently, namely the study of gas exchange, which gives vital but only integral values for the organism as a whole, and the study of tissue respiration on tissue homogenates and tissue sections.

The literature pertaining to this question is referred to in detail in Suezhko's papers. It should be pointed out that when investigating total gas exchange somewhat contradictory data are obtained which are evidently associated with the conditions of treatment, the time of observation and the individual behaviour of various animal tissues.

As to sections and tissue homogenates, as well as to simpler objects, such as sea urchin eggs and plants, here an inhibition of tissue respiration is observed as a rule. From this viewpoint the data obtained in our laboratory are in agreement with the observations on tissue respiration *in vitro*. The advantage consists, however, in that, as we shall see below, this method allows the possibility of following up tissue response *in vivo* and of sufficiently distinctly determining the curve of this response against time.

Most distinct results are obtained with the aid of the "oxygen test." The curve of an increase in diffusion current in response to the inhalation of a dose of oxygen is determined several times for each experimental animal. This curve is, as a rule, quite typical and stable in its shape and amplitude for each given animal and for a definite position of the electrodes.

After total irradiation with a dose of the order of 1,000 r, or after local irradiation to the head with doses from 1,000 to 3,000 r, the diffusion current starts to increase some 5 to 10 minutes after irradiation. The maximum of this increase,  $\Delta I$ , is reached two to three hours after exposure. The curve rises and the time at which it begins to fall, i.e. the time of utilization, increases conspicuously, up to twofold and more. The time of utilization is often disproportionately prolonged, much more than the amplitude increase.

Taking into consideration that this peculiar change of the reaction proceeds against the background of progressive haemodynamic disturbances, it can be ascribed only to a deterioration of the ability of the tissue to utilise oxygen.

Of key significance is the fact that this reaction is by no means a result of complex secondary or tertiary changes, and bears witness to the direct local response of the tissue. In more general form this was demonstrated by us upon a change in the localization of the action.

Shielding of the head and irradiation of the abdomen leads to reverse pictures in the behaviour of the "oxygen test" which point to deficiency of free oxygen at the root of haemodynamic disturbances. Compared with the control, prior to irradiation, oxygen dosage causes no increase; moreover, it brings about a clear decrease (Fig. 2). The additional amount of oxygen is greedily utilized by the tissues of the irradiated organism.

Indeed, no concept of activation of oxidative processes arising in the central nervous system following abdomen irradiation is required here. It suffices to perceive clearly that a tissue is able to cover a deficiency associated with a disturbance of blood circulation at the expense of an additional portion of oxygen.

The following form of the experiment, however, seems to demonstrate still more convincingly the local and direct character of the response produced by radiation. The brain was irradiated through an aperture

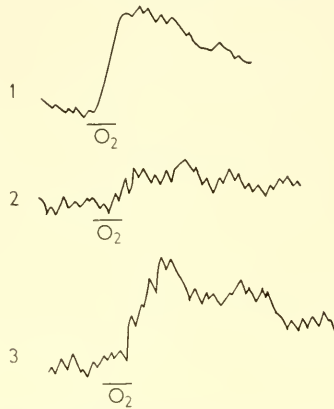


Fig. 2.—Change  $\Delta\tau$  in the result of abdomen irradiation.

1, before irradiation; 2, 2 hr after irradiation; 3, 3 hr after irradiation.

in the lead screen as small as 4 mm<sup>2</sup>, the entire body and the remaining part of the head being screened. At the site of the treatment two electrodes were inserted, one of them into the cortex, the other into the

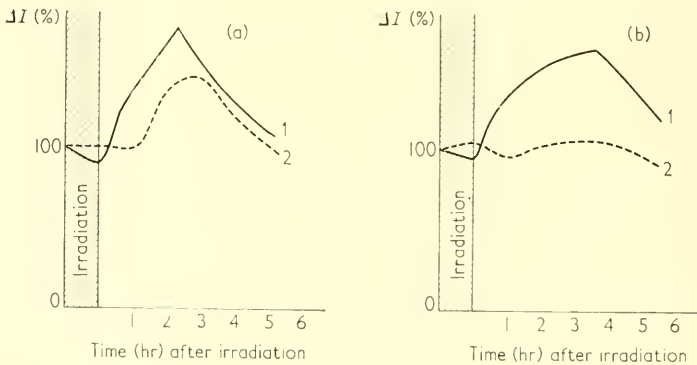


Fig. 3.—Dynamics of the change  $\Delta\tau$  in the irradiated and unirradiated area of the brain. (a) subcortex; (b) cortex. The value  $\Delta\tau$  is a percentage of the norm taken as 100.

1, irradiated area;

2, unirradiated area.

subcortex. Another pair of electrodes was arranged at a distance of 5 to 8 mm from the small irradiated area of tissue. When recording the "oxygen test" only in this small irradiated area an increase of the

diffusion current both in the cortex and subcortex was found (Fig. 3.). This proves a deterioration of the ability to utilize oxygen. Control electrodes showed either no changes or the changes were much smaller and, which is most important, those which arose did so with a considerable delay. The latter fact shows that the given attenuated effect arose at a distance of some millimeters not at the expense of radiation dispersion in tissues but as a peculiar "diffuse" distribution of the inhibition of oxidative processes on account of specific substances formed at the site of treatment. This is confirmed by the fact that a decrease in the dose of irradiation to 100 r, when the "oxygen test" no more gives statistically trustworthy results, leads only to a diminution of the amplitude of this effect without causing a delay in time.

Thus we see this method a possibility of directly identifying primary cellular responses to radiation under *in vivo* conditions in a complex organism.

One more interesting phenomenon turned out to be related to these apparently local and primarily-arising responses to radiation.

A thorough analysis of the regularities of the behaviour of free oxygen in tissues showed its level to be far from stable in normal conditions. Actually in all cases a variation in this level is observed, i.e. a rhythm of oxygen content in the tissues. These rhythmical changes are mostly of two orders. A rhythm of a smaller amplitude and a higher frequency, 15 to 20 per min, and a rhythm of a greater amplitude with a frequency of 2 to 3 per min. Very often both rhythms existed simultaneously (Fig. 4.).

This rhythmical free oxygen level turned out to be functionally related to the state of the central nervous system. So, oxygen rhythm is probably eliminated upon experimental development of the inhibition

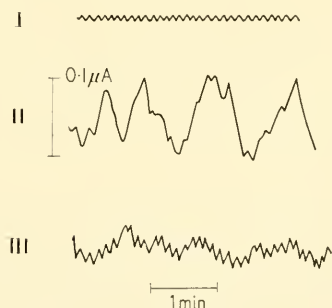


Fig. 4.—Forms of rhythm of oxygen tension in the brain.

- 1, rhythm with the frequency 15 to 20 per min;
- 2, rhythm with the frequency 2 per min;
- 3, The first and second forms of rhythm combined.



process, being considerably changed in different ways under the action of pharmacological agents, drugs in particular.

Now a very difficult question arises: what determines the rhythmical character of the oxygen content. On the one hand, the point can be the rhythmical variation of local blood circulation, the vasomotor periodicity. On the other hand, the rhythm of oxidative processes themselves cannot be ruled out.

Forestalling the events, it should be pointed out that, having no possibility for the time being of rejecting the first cause, experiments carried out on an entire animal lead us towards the acceptance of the important role played by fluctuation of the rates of oxidative processes.

This is based on the fact that the rhythmical character of the free oxygen level can be observed in a metabolizing tissue not only when blood circulation is cut off but also in several objects lacking this latter completely.

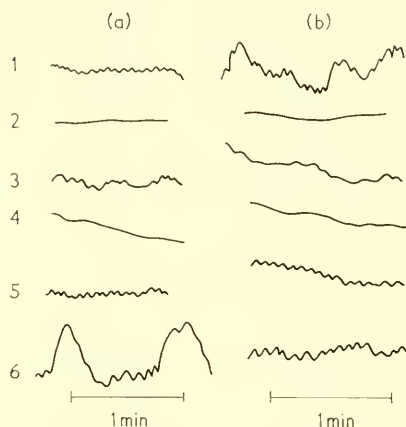


Fig. 5.—Change of rhythm in the rabbit brain as the result of head irradiation. (a) cortex; (b) subcortex.

- |                            |                             |
|----------------------------|-----------------------------|
| 1, before irradiation;     | 4, 3 hr after irradiation;  |
| 2, 10 min. later;          | 5, 6 hr after irradiation;  |
| 3, 1 hr after irradiation; | 6, 24 hr after irradiation. |

With a change in free oxygen level in tissue after irradiation and with a change of the curve of "oxygen test", disturbances of this rhythm are also observed. In many instances, 8 to 10 min after termination of exposure the rhythm disappears completely both in the cortex and in the subcortex (Fig. 5). The restoration of this rhythm proceeds in a different fashion. As a rule, the quicker oscillations are restored after 30 min, while the depression of the slower ones can be preserved for a

long period. Radiation seems to hit some link of rhythmical activity connected with oxidative processes of rapidly manifested effect.

Restoration of rhythmic activity in time does not coincide with that of the ability to utilize oxygen. Though both processes are interrelated to some extent, this interrelation is neither rigid, nor simple.

It is very interesting that rhythmical fluctuations of oxygen content can be revealed under certain conditions in isolated pieces and sections of organs of a homoiothermal animal, for example, in a liver.

The same phenomena are found in plants and even in a population of yeast cells.

Distinct rhythmical changes in free oxygen content and disturbances of this rhythmical activity were obtained with the roots of *Vicia faba* seedlings. Under normal conditions, with an appropriate electrode insertion, not very regular and ordered changes in the level of oxygen content are clearly revealed. In some cases periods with a great amplitude of the order of 1 per min or even 1 per 1.5 to 2 min and those of the order of 10 per min appear.

When the accumulated radiation dose reaches some hundreds of r, an appearance of a kind of inhibition of this rhythm, arising almost immediately after irradiation is very characteristic (Fig. 6). In the process of irradiation this rhythmic activity in the form of irregular splashes is manifested from time to time by either a small or a very sharp and considerable change in the level of oxygen content. By the end of irradiation with a dose of 10,000 r, (which is not an excessive one for plants), a practically straight line appears instead of a curve of dentate character. The change takes place 1 hr after exposure, while after more than 2 hr the absence of any oscillation is still more clearly manifested. Thereafter a continuous drop of the total level of free oxygen content takes place. So, prior to irradiation this level amounts to 6 to 7 "units". By the end of the irradiation it has fallen to 3.5 units. After 3 hr with the most smooth character of the curve and a complete absence of the rhythm this level becomes 2. It then starts to be gradually restored.

It can be seen that 7 hr after exposure the rhythmical character of the curve is recovered; after 24 hr it is manifested rather distinctly, while after 2 to 3 days rhythmical activity is clearly stimulated. Amplitude and, above all, frequency of the rhythm, are much greater than they were prior to exposure.

A decrease of the total level of free oxygen at the moment of the most practically complete smoothing of the rhythm does not contradict the facts mentioned above and observed in the tissue of the central nervous system of a living experimental animal. In this case the level of free

oxygen naturally depends on the rate of diffusion from the external medium and it may be supposed that the effect of the radiation which leads to a depression of the rhythm simultaneously disturbs conditions

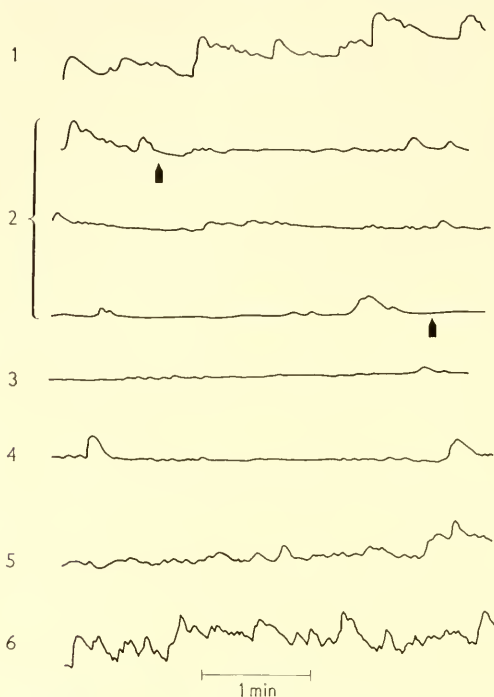


Fig. 6.—Change of rhythm in a bean root as the result of irradiation. Dose, 10,000 r.

- 1, before irradiation;
- 2, during irradiation (the arrows indicate the beginning and the end of irradiation)
- 3, 3 hr after the end of irradiation;
- 4, 5 hr after the end of irradiation;
- 5, 7 hr after the end of irradiation;
- 6, two days after irradiation.

of oxygen diffusion from the outside. The question, however, is not only one of diffusion transfer, but also of oxygen circulation phenomena associated with the continuous motility of the protoplasm—the protoplasmic streams.

When comparing free oxygen level and rhythmic activity, one can consider the depression of rhythmic activity to be an indirect result of a decrease in free oxygen level. This is proved by the very start of the effect and measurements taken directly under the beam. Depression of rhythms sets in, practically at the very first minute after irradiation when the free oxygen level has had not time to decrease. A decrease of

this level by about 1.5 times takes place only by the end of the irradiation when the accumulated dose reaches 10,000 r. The drop of free oxygen level down to its minimum takes place not earlier than 3 hr after irradiation termination.

In another experiment with an exposure of 3,000 r, the primary, irregular, but distinct, rhythm is inhibited by the time of termination of the exposure. A complete arrest is observed after one more hour, while after 3 hr the rhythm is gradually recovered.

In this case both the inhibition of the rhythm and its restoration are observed without any change in the general level of free oxygen content. An impression is gained that the disturbance of rhythmic processes is related to some earlier functions than a change in the total level of oxidative processes and, therefore, of free oxygen content.

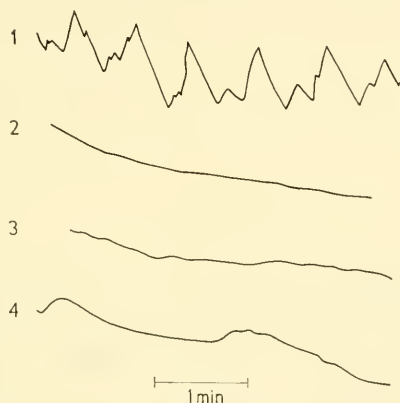


Fig. 7.—Change of rhythm of yeast cells as the result of irradiation. Dose, 5,000 r.

1, before irradiation;

2, 3 min after irradiation;

3, 1 hr after irradiation;

4, 1.5 hr after irradiation.

It is noteworthy that this inhibition of rhythmic activity, as well as the change in free oxygen level proceed in a different manner in different parts of a plant. It is particularly characteristic of the growing root. According to available data, the stems or leaves of a plant which possess a higher radiation resistance do not show an immediate depression with lower doses than those mentioned; moreover, with the dose of 2,000 r they show even a change and increase of the rhythm, as if radiation stimulates, and an appearance of a more distinct and regular component of rhythmical activity.

A very distinct cessation of the rhythmical process can be observed with an intensely growing yeast culture in a liquid medium (Fig. 7).

The condition for the rise of this rhythm is a good start of the development of the whole cell population. Then the clearly manifested, sometimes extraordinarily demonstrative and very regular, oscillating character of the free oxygen level is revealed. Some minutes after irradiation a quite smooth line is seen instead of the dentate one registered prior to exposure. In other cases, under similar treatment the character of the rhythmical process is conspicuously changed. Regular waves of a change in free oxygen concentration are replaced by an irregular broken line.

The slow rhythm of the rate of oxygen utilization is by no means an exception. Two other types of periodic phenomena which were found recently in our laboratory are worth mentioning.

The first is the slow component of bioelectric activity, from the work of Dr. Aladjalova. It turns out that, apart from electric processes of impulse character, with the frequency of several times per second and a steep augmentation of the difference of potentials, a very slow rhythm can be revealed in many objects. This was called by Dr. Aladjalova, (1956), the "infra-slow" component of the change of potential in direct current.

The regularity of such a rhythm is clearly revealed, for example, in various recordings from the tissue of the central nervous system. This form of electric activity is also most closely related to functional state.

Consideration of this question in detail exceeds by far the problems concerned in our conference; therefore, I shall give but one example.

Under defined conditions a depression of infra-slow oscillations of the potential in the brain cortex after irradiation of the rabbit head, say, with 1,000 r can be clearly shown. A not very regular rhythm of the order of 8 to 10 oscillations per minute becomes smoothed 5 min after irradiation. Twenty-four hours after irradiation this rhythm is restored and even acquires a greater amplitude and regularity.

From its appearance only—and for the time being we can only speak of this—the very character of the process, its depression and in some cases even a stimulation of rhythmical activity some time after irradiation very much resemble all that has been said with respect to the periodic character of the processes of oxygen utilization.

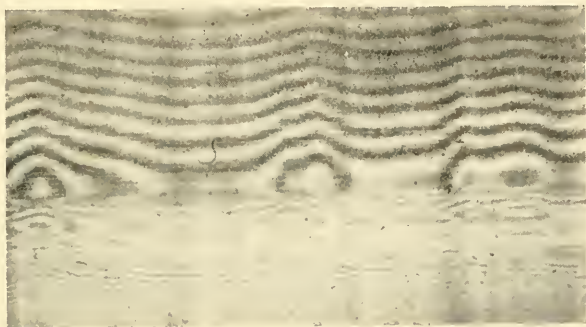
We have failed thus far to establish the complete synchronism of both processes upon their simultaneous registration. They run simultaneously and with comparable frequencies while being determined by different physico-chemical mechanisms.

The possibility of observing periodical mechanical motility in cells and tissues not specialized for the function of motility seems to us to be of no lesser importance. This mechanical motility can be revealed



only by the sensitive interference method able to register mechanical shifts of some fractions of a micron which lie beyond the resolving power of an ordinary microscope.

Thus, when observing the surface of an isolated cricket ganglion, periodic oscillations can be revealed by a shift of interference bands, which approach by their frequency the characteristic slow processes of oxygen utilization or oscillations of direct current potential (Fig. 8(a)).



(a)



(b)

Fig. 8(a).—Interference lines for the surface of the active ganglion of the cricket;  
(b) Interference lines for the inactivated ganglion surface.

This structural motility is also correlated with the functional state. It is cut off under anaesthesia, or on the loss of viability, or upon any injury, in almost the same way as was shown for simple objects in respect of the rhythm of oxidative processes. By now the comparison of the ultrastructural motility with other periodical phenomena proceeds by the outward appearance only. A judgment of the extent

of their inter-relation requires the development of procedures of a strictly spatial matching and observation of these phenomena.

When discussing the meaning of these periodical processes and their damage by radiation we can but voice some speculations.

The thesis that perfect mechanisms of self-regulation must exist in the chemical activity of living cells is irrefutable. Self-regulating mechanisms must be flexible and, in the terms of modern techniques, must at any given moment provide the adjustment of all the running processes to the most advantageous working regime.

In respect of most chemical processes it would be very unlikely to suppose that they maintain either their level or their rate, changing smoothly so that the curve of these changes in time will be of a monotonous character.

Any regulator has tolerance, and the regulatory process fluctuates between both the upper and the lower limits. It is just according to this principle of relay or impulse regulation that the most perfect, automatically working, self-regulating and self-adjusting systems are constructed.

This type of regulation for which modern techniques have only recently been available is supposed to have been worked out millions of years ago in the process of evolution. The level of oxygen processes, which is of dentate character, may witness this kinetic regulatory process in its outward appearance.

What are the possible mechanisms for such a regulation? We need another one, even though somewhat fantastic assumptions are required. The existence of a continuous relationship between the structural organization of cells at any given moment and kinetic exchange processes is beyond any doubt. This viewpoint is confirmed by all the recent findings when the problem of the intermolecular architectonics of living has become so developed and received such a new significance; when the dynamic aspects of morphology are being developed and the continuous structural mobility at any level of the organization of living organisms is being more and more demonstrated.

For this the specific structural and ultrastructural organization not only provides a co-ordinated running of chemical processes but on the contrary this co-ordinated running maintains the very structural organization. On the cessation of vital activity a disorder between structural organization and the interacting ensemble of chemical processes takes place.

This inter-relation is of a typical character of reverse relation, i.e. of interaction absolutely necessary for the construction of any self-regulating system.

It should not be said that the given principle of interrelation between structure and exchange processes is the only mechanism of self-regulation. A living system is so reliably self-regulating with respect to usual technical constructions because it undoubtedly has many various reserves and spare channels in regulatory mechanisms.

However, such a distinct relation between structure and exchange processes gives us the possibility of considering it with rather firm conviction to be a self-regulating mechanism of no small importance.

It is from this viewpoint that we compared rhythmical structural motility and rhythmical processes of oxygen utilization.

By now the running of definitely localized chemical processes in time has not been fixed by procedures of direct recording. The facts we observe when we employ polarographic sounding may be the first step in this domain. From the other side we know how phenomena of motility in living systems are distributed. A continual change in the forms of cells and an interchange of their separate elements takes place. Currents of plasma are continuously running. Mitochondria contract and relax, cell nuclei pulsate and in some cases this structural motility in the submicroscopical range as was shown, is of the character of a more or less rhythmical process. Moreover, hardening of structure is the first and most convincing feature of death from its appearance.

All this leads us to suppose that structural motility is one of the most important elements of the regulation of kinetic cellular processes, (Frank, 1958a, b; 1960).

If it is so the problems stated above must be considered apart from the undoubtedly important problems of the effect of radiation on large-molecular components, upon enzyme systems (both isolated and *in situ*) and directly-forming structural organization. The question is here of an influence not on separate links and components but upon the interrelation between structure and exchange processes which play a big role in the ordering of chemical processes in a cell, the co-ordination of all running processes.

In this manner new ways of understanding the reinforcing mechanisms which determine radiobiological reactions in living cells may be found.

Thus the question is of an analysis of the influence on regulatory mechanisms and of the damage that takes place in the main mechanism of self-regulation, the system of reverse relations—interrelation between structures and exchange processes.

If the rhythm of phenomena is thought of as an external manifestation of a working regulatory mechanism, we can see a possibility of taking one step more in the direct experimental analysis of an inhibition

of this regulation mechanism by studying kinetic phenomena—the rhythm of oxygen utilization and the rhythm of submicroscopical motility.

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#### DISCUSSION

MANOILOV: Does the rhythmicity which you have so well shown, occur throughout the radiation sickness or only during the first hours? Did you observe such rhythmical changes in other tissues of the living organism?

FRANK: Today I have only spoken about the first hours, but this change of the free oxygen balance occurs throughout the long period of the reaction in the irradiated organism. But, at later stages, these changes are of a secondary nature. Similar changes of the relationship were observed on very different objects. Today I have demonstrated as examples only plant material and yeast.

SHABADASH: The rate of oxygen utilization depends on the enzymatic activity of mitochondria. You know, that in our laboratory it has been shown that within a few minutes of irradiation cytochemical changes occur in the nerve cell mitochondria of the brain cortex. What data can you give on electron microscopic changes of the mitochondria, which parallel the changes in oxygen utilization?

FRANK: Because of time limitations I was not able to dwell either on your work or on the very interesting work of Noed, who has shown that practically immediately after the irradiation the number of mitochondria, determined in liver mitochondria fraction, rapidly decreases. It is remarkable that this process is reversible, the numbers later returning to normal. The number of mitochondria is restored within about 5 to 6 hours, showing good correlation with the time course of the oxygen utilization disturbances which I have demonstrated here.

BACQ: In the experiments in which oxygen tension was studied in living tissues the tip of the electrode was in contact with the cell, but was not within the cell. What was the position of the electrode when you worked with the yeast cells?

FRANK: In case of yeast the electrode was immersed in a drop of the yeast culture.

ZELISCHEV: It is remarkable that when a leaf is irradiated, there was no local effect as was observed with all other objects. If it is an extraordinary exception from the general rule, very interesting consequences may follow. Please, explain in detail your views on this matter.

FRANK: The effect was also local but in this case, as is often the case with other

objects when smaller doses are administered, it produced no immediate depression, but radiation irritation i.e. a reverse reaction. I believe that if the radiation dose given to the leaf had been increased then, taking into account the small sensitivity of the developing leaf, we would have obtained an identical effect.

ERRERA: Would it not be possible to link the rhythmicity in the cell's oxygen tension in the experiments with brain with the heart's rhythm?

FRANK: Of course. When we worked with living warm-blooded animals naturally the question arose as to whether it would be possible to link this rhythm with that of the circulation. It could not be compared to the heart's rhythm, they differ by their frequency, but the question arose whether this rhythm could not be the effect of the play of the vessels, the vessel's contractions. Of course, this question could not have been solved if we had not worked with isolated pieces of the tissues, with isolated liver and other objects, in which there is no circulation.

BLUMENFELD: Did you study the effect of temperature on the rhythm?

FRANK: We studied the temperature effects on rhythm, of course not on warm-blooded animals but on simple objects—developing frog eggs, plants and so forth. In the effects of the temperature on the rhythm there are clear-cut regularities. We also studied effects on the oxygen supply.





# IMMEDIATE REACTIONS OF NERVES AND MUSCLES TO IONIZING RADIATION

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## SUMMARY

In contrast to the peripheral motoric nerves and striated muscles but similar to smooth muscle organs of vertebrates nerve-muscle preparations of worms show immediate partially reversible reactions to relatively low doses of X-rays as changes in motility and contraction. Nerve-muscle preparations are more sensitive than isolated muscles. Remarkable is the strong dose-rate dependence of these functional changes. There are some indications that the effects are due to reversible changes of cell permeability under and shortly after irradiation.

## INTRODUCTION

On earlier occasions (Hug, 1958, 1960) I have had the pleasure of presenting a film showing that many lower animals react to ionizing radiation with reflex-like motions: snails retract their tentacles; clams retract their gills and close their shells; actiniae incline and shorten their tentacles and subsequently the whole body of the animal is retracted and shortened. The leech shows peristaltic contractions, writhes violently and finally moves out of the X-ray beam. The small sea urchin, when irradiated under water, retracts its feet and moves out of the irradiated area too. A crustacean, the barnacle, stops the rhythmic opening and closing of its cover and the grasping motions of its cirripedia. Many insects, such as ants, spiders and fleas, show characteristic behavioral changes under X-irradiation. The same effects as with 50 kV X-rays have been produced by  $\alpha$ -rays in some of these animals. Those of these effects which we have investigated quantitatively show a characteristic dependence on dose and dose-rate (Hug, 1959). In each species, a minimum dose-rate is necessary to produce the effects under continuous irradiation. Above this threshold the minimum irradiation time decreases with increasing dose-rate. The product of dose-rate and necessary irradiation time is not constant over the whole range of dose-rate. In this respect there exists at least an

analogy to the well-known strength-duration curve of the reaction to electric stimulation. A similar dose and dose-rate relationship has been found in the immediate contraction of mammalian intestine under irradiation (Hug, 1960; Schliep *et al.*, 1961). The threshold of dose and dose-rate varies considerably in different species and organs, and even in individuals of the same species.

However these observations did not permit us to draw definite conclusions with regard to the mechanism of action underlying these immediate radiation effects nor to define the site of action. Behavioral changes of *Daphniae* under X-irradiation observed in the meantime by Baylor and Smith (1958) are evidently due to effects on the Nauplius eye of these animals. Therefore it must be taken into consideration whether or not some of the effects observed by us are due to a similar stimulation or disturbance of photoreceptors and may be compared with the so-called roentgenphen in higher animals, recently investigated by Lipetz (1955) in a very careful manner. Evidently not all effects observed by us can be attributed to stimulation of photoreceptors but must be related to irritation of other parts of the nervous system or other tissues. The reaction of a living animal as a whole, however, seemed to be too complicated to clarify this question.

### EXPERIMENTAL

Recently we have used isolated systems, e.g. nerve-muscle preparations to study the different parameters on which such immediate effects may depend. Nerve-muscle preparations of the leech (*Hirudo med. L.*) and of the earthworm (*Lumbricus terr.*) and isolated muscles of the leech proved to be very sensitive to radiation and suitable for detailed studies.

Isolated strips of the ventral body wall still connected with the nerve cord or isolated muscles, of both the species, were suspended vertically to a lever, which registered the isotonic contractions on a smoked drum. The preparation was kept in a moist chamber, rinsed continuously by a modified Ringer-solution and aerated with air or oxygen or a mixture of 95 per cent oxygen and 5 per cent CO<sub>2</sub>. The specimens were irradiated in this position with 50 kV X-rays filtered by the beryllium window of the tube only. The doses and dose-rates given below are measured in air at the site of the specimen.

#### *Nerve-muscle preparations*

Two different effects could be observed: in a nerve-muscle preparation showing spontaneous contractions irradiation can inhibit these contractions temporarily (Fig. 1) and conversely, in a specimen that is

relatively quiet irradiation can induce rhythmical contractions, which cease after a certain time (Fig. 2). The average tonus rises as in Fig. 2 or falls as in Fig. 1(a) and (b). In analogy with reflex-like reactions of lower animals and of rabbit intestine as described above, there is a striking dependence of the effects on the dose-rate and furthermore on the total dose. Until now, the lowest effective dose-rate was found to be about 100 r/min in the nerve-muscle preparation of the earth-worm; 15 sec of irradiation proved to be sufficient to elicit the effects and therefore the minimum necessary dose was some 25 r. These threshold values, however, are dependent on the sensitivity of the instrumental arrangement and should not be considered as true threshold values in a toxicological sense. The latent period between the start of irradiation and the beginning of the reaction lies in the range of a minute at the lowest dose-rates and shortens with increasing dose and dose-rate to non-measurable values.

#### *Muscle preparations*

In order to differentiate the radiation effects on nervous elements from those on muscle fibres, we investigated strips of the ventral body wall of the leech which were completely freed from the nerve cord. This isolated muscle reacts within seconds to irradiation too. However the doses and dose-rates necessary to produce these effects were considerably higher than in the case of intact nerve-muscle preparations. Depending on the experimental conditions this radiation-induced contraction may be either partially or completely reversible or it may be irreversible. Normal muscles, suspended in a Ringer-solution between pH 7 and 8 saturated with air or oxygen, show an almost complete relaxation following a radiation-induced contraction. Only after high doses can a residual contraction be observed, and this increases with the total accumulated dose.

Relaxation of muscles following the contraction can be prevented completely by suspending the specimen in a more acid solution (about pH 6), or by adding 5 per cent  $\text{CO}_2$  to the aerating gas mixture, or by certain metabolic inhibitors e.g. dinitrophenol (9 mg/l). Under these experimental conditions, irradiation will produce a permanent state of contraction, persisting usually for hours, without any relaxation. If, however, after irradiation the medium is exchanged for a more physiological one the ability to relax is restored.

#### *Irradiation-induced contraction in a muscle unable to relax.*

Under continuous irradiation with a constant dose-rate the contraction of such a muscle follows first an S-shaped curve and then a

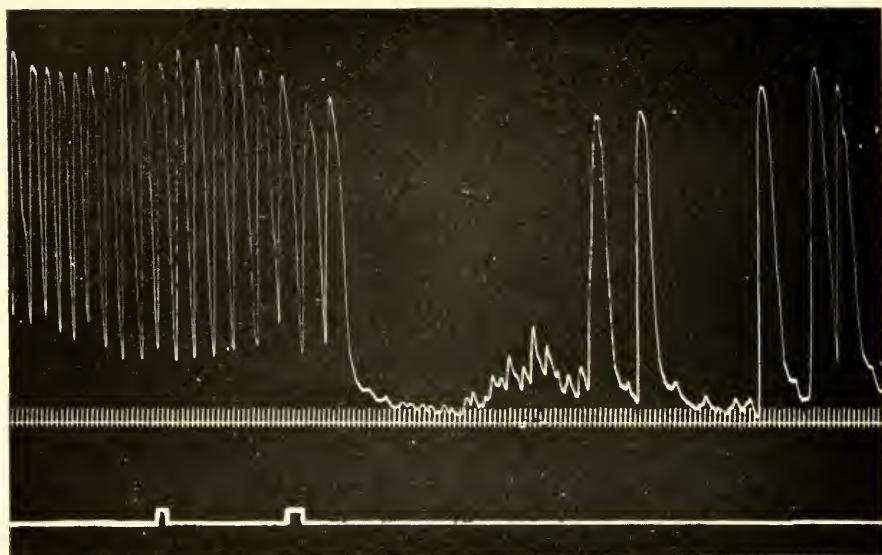


Fig. 1a

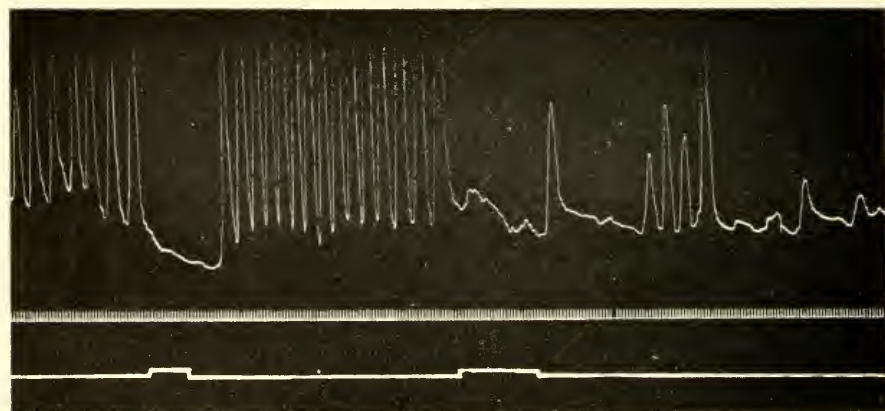


Fig. 1b

nearly linear curve with a much flatter slope (see Fig. 3(a) and the first part of 3(b)). With the arrangement used, a significant contraction could easily be registered under irradiation with a dose-rate as low as 330 r/min. The increase of the dose-rate shortens the interval between the start of irradiation and the beginning of a visible contraction from about a minute to seconds, the slope of the S-shaped and the quasi-linear



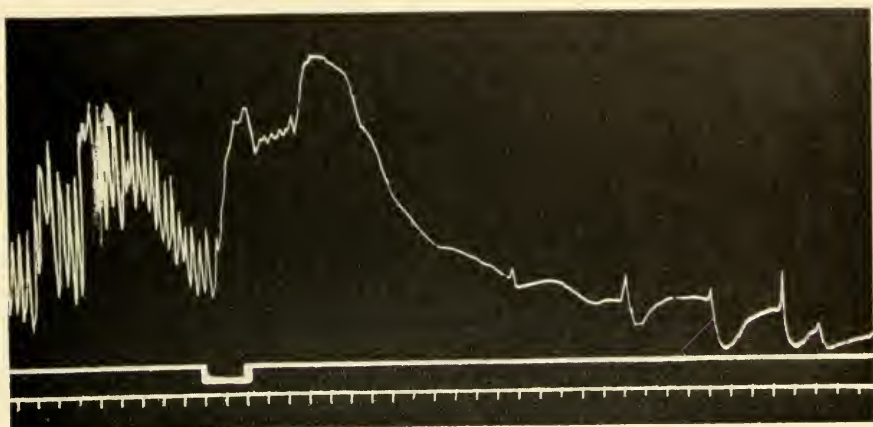


Fig. 1(c)

Fig. 1.—Nerve-muscle preparations. Cessation of spontaneous rhythmic activity after X-irradiation.

(a)—Earthworm: 115 r/min. Irradiation time 10 sec (without effect) and 15 sec. Time marks: 5 sec.

(b)—Earthworm: dose-rate 4,000 r/min. Irradiation time 1 and 2 min. Time marks: 5 sec.

(c)—Leech: dose-rate 13,200 r/min. Irradiation time 2 min. Time marks: 1 min.

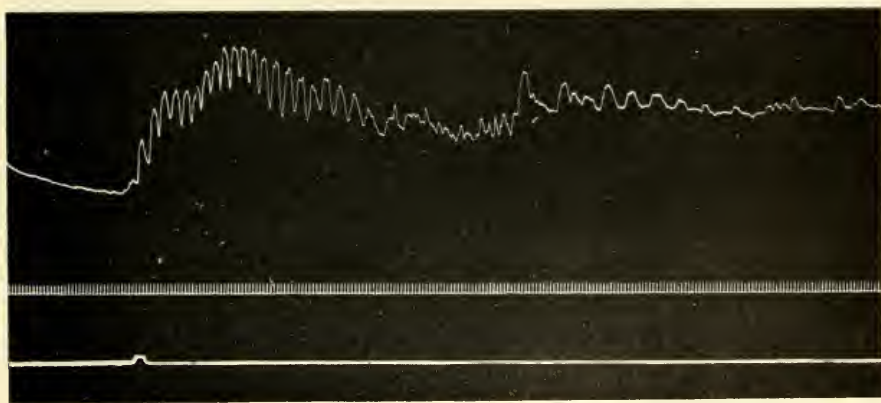


Fig. 2.—Nerve-muscle preparations of the earthworm. Induction of rhythmic motion and increase of average tonus by irradiation with a dose-rate of 100 r/min. Irradiation time 15 sec. Time marks: 5 sec.

part of the curve becomes steeper and the plateau which the sigmoid part of the curve tends to approach becomes higher. Under irradiation with very high dose-rates the sigmoid part of the curve reaches a real plateau. The latter corresponds to the largest possible contraction of the muscle amounting to about 55 per cent of the initial

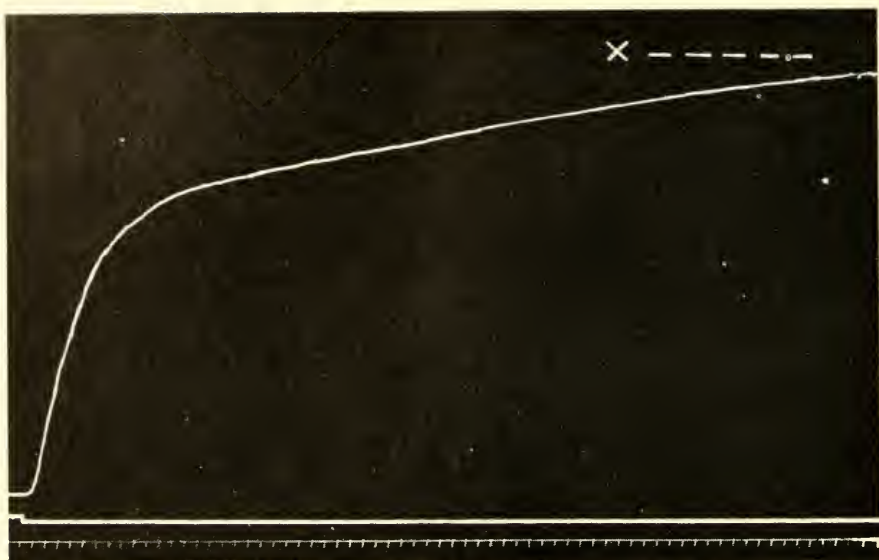


Fig. 3(a)

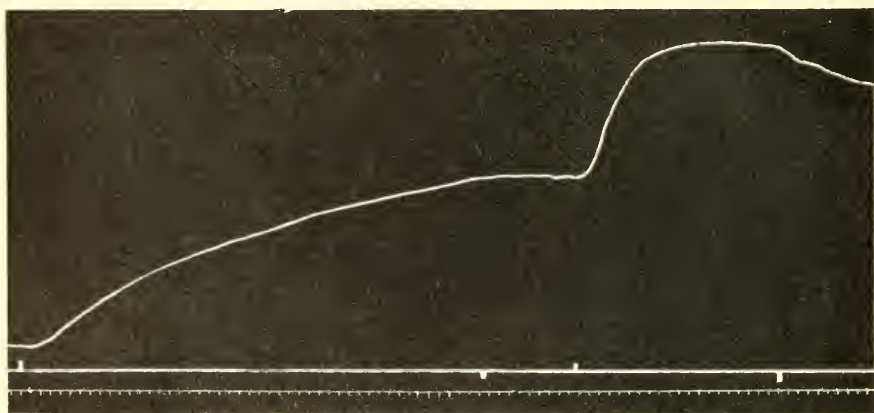


Fig. 3(b)

FIG. 3.—Contraction of leech muscles under continuous irradiation.

(a) In Ringer solution aerated with 95 per cent  $O_2$  and 5 per cent  $CO_2$ . Dose-rate 6,600 r/min, Irradiation time 120 min. Time marks: 1 min.

x — — — — final length after 2 hr irradiation.

(b) Consecutive irradiation with two different dose-rates, firstly with 3,300 r/min to 46 min and secondly with 26,400 r/min to 20 min.

During the first irradiation period the medium was aerated with 95 per cent  $O_2$  and 5 per cent  $CO_2$ .

Shortly before the second irradiation the medium was exchanged for a solution aerated with pure oxygen, therefore the muscle relaxed after the second irradiation.

↑ start and ↓ end of irradiation. Time marks: 1 min.

length as may be seen in the second part of Fig. 3(b). The curves in Fig. 3 demonstrate also the dependence of the reaction on the medium. During recording of the curve (a) and the first part of (b) the muscle was kept in a medium preventing relaxation. Shortly before the second irradiation in Fig. 3(b) was started the medium had been changed i.e. the muscle was then rinsed with a solution aerated with pure  $O_2$ . Therefore the muscle could relax after the second irradiation.

A single exposure to a submaximal dose produces a partial shortening of the muscle. Again the latency time and the slope of the contraction curve are determined by the dose-rate, whereas the final degree of shortening is determined by the dose-rate as well as by the duration of exposure. The process of shortening stops very soon after irradiation and the muscle persists in this state of partial contraction. Under repeated exposures the muscle contracts in a step-wise fashion (Fig. 4).

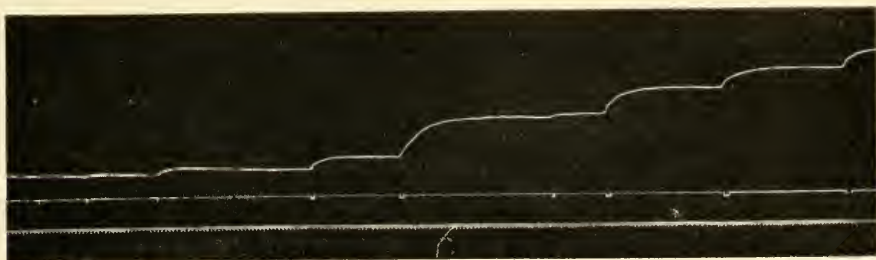


Fig. 4.—Repeated irradiation of leech muscle in Ringer solution, aerated with 95 per cent  $O_2$  and 5 per cent  $CO_2$ .

Irradiation pulses (from left to right) of 10, 15, 20, 25, 30, 10, 35, 40 and 50 sec.

If the final degree of the contractions after each radiation pulse is plotted against the accumulated dose sigmoid like dose-effect-curves result. If one increases the dose-rate at which the single impulses are delivered, the slope of the dose-effect curve becomes steeper and the degree of final contraction increases as well (Fig. 5). The importance of dose-rate becomes especially evident if one irradiates the same specimen repeatedly with identical doses but at different dose-rates (Fig. 6).

#### *Irradiation-induced contraction in a muscle that can relax*

In a medium where the metabolic processes are practically undisturbed the muscle is able to relax after the radiation-induced contraction. Figure 7 shows the effect of repeated irradiation with the same dose-rate but with increasing doses. The latency time in all cases is practically the same and the slope of the contraction curve too. The maximum of contraction after each irradiation depends on the irradiation

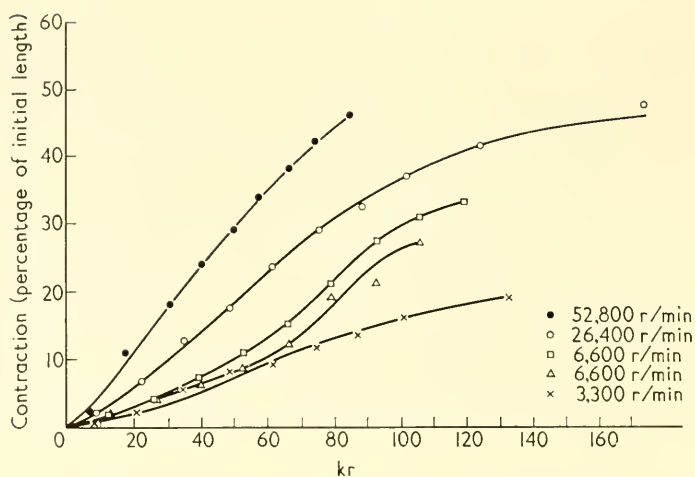


Fig. 5.—Diagram of cumulative shortening of leech muscle (aerated with 95 per cent  $O_2$  and 5 per cent  $CO_2$ ) under repeated irradiation with various dose-rates.

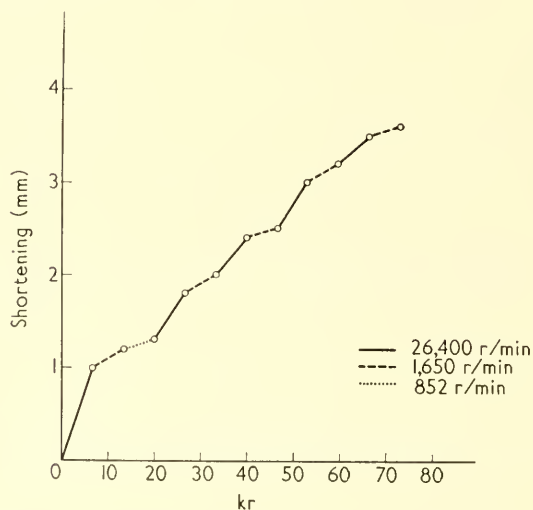


Fig. 6.—Cumulative shortening of a leech muscle unable to relax under repeated irradiation with the same dose of 6,600 r, delivered at different dose-rates;

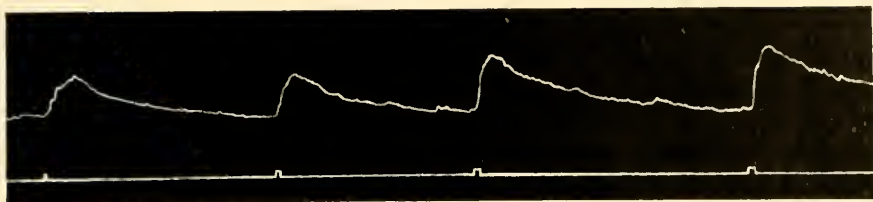


Fig. 7.—Leech muscle rinsed with Ringer solution at pH 8.0, aerated with  $O_2$  only. Repeated irradiation with a dose-rate of 10,000 r/min and irradiation times (from left to right) of 10, 20, 25 and 30 sec.

time, i.e. the dose. Shortly after irradiation the relaxation becomes apparent and seems to follow an approximately exponential curve. Repetition of the irradiation and thus increasing the accumulated total dose results in a residual contraction which becomes more and more prominent. The rate of relaxation diminishes with the accumulated dose.

If such a normal muscle is irradiated continuously with suitably chosen dose-rates a kind of steady state of contraction is produced (Fig. 6). If, during irradiation with a certain dose-rate, no further contraction occurs, then irradiation with a higher dose-rate induces an additional shortening of the same preparation until a higher degree of final contraction is reached (Fig. 8).



Fig. 8.—Leech muscle (rinsed with Ringer solution at pH 8.0;  $O_2$  saturated). Consecutive irradiation with two different dose-rates. Under irradiation with a dose-rate of 594 r/min for 77 min a steady state of contraction is reached. After irradiation ceases the muscle relaxes. The following irradiation with a dose-rate of 4,000 r/min produces a steeper contraction curve and a higher final contraction. See also the difference in latency time.

↑ start and ↓ end of irradiation. Time marks: 30 sec.

## DISCUSSION

The radiation-induced contraction seems to be a complex process in which at least two components may be distinguished: namely an immediate contraction, which is reversible if the metabolic processes



of the muscle are undisturbed under irradiation or if they are restored after irradiation, and a contraction which is irreversible over many hours under all conditions examined by us. This irreversible part of the contraction can be derived from the nearly linear part of the contraction curve following the initial S-shaped curve under continuous irradiation in Fig. 3 and from the residual contraction in Fig. 7. Its dose response is very much lower than that of the reversible contraction.

The following discussion deals mainly with the immediate recoverable reaction hitherto unobserved. Studying it quantitatively we would, however, have to subtract the contraction curve from the irreversible residual contraction.

A particularly striking phenomenon is the marked dependence of the radiation-induced reversible muscle contractions on the distribution of dose with time. Not only the latent period and the slope of the contraction curve but also the final contraction under continuous or repeated irradiation is determined by the dose-rate. This holds true especially in muscles with undisturbed metabolic properties. Here the tendency of the muscle to relax counteracts the contraction-producing effects of radiation. But also in muscles in which relaxation is inhibited a dose-rate dependence exists as shown in Fig. 3 by the different levels which the sigmoid parts of the curves tend to approach, and in Fig. 5. Therefore at least two different mechanisms must be responsible for the dose-rate dependence: firstly an unknown process counteracting the primary physico-chemical changes in the cell which produce contraction, and secondly the relaxation tied to metabolic properties. As seen in Fig. 8 under not too high dose-rates a "steady state" of contraction can be reached, which must be considered as the result of the ratio of dose-rate on the one hand and the two counteracting processes on the other.

Thus in a two-fold sense the muscle acts as a sort of "biological dose-rate meter", operating badly, however, because of the changes of its characteristic properties with increasing dose, i.e. the increasing residual contraction and the decrease of relaxation rate.

In our current experiments we are attempting to elucidate the mechanism of action of the radiation-induced muscle contractions.

Treatment of muscles with glycerol destroys cell membranes but leaves the contractile muscle elements intact and responsive to ATP. In preliminary experiments muscles thus treated and then exposed to X-rays failed to contract. This would exclude a direct effect of radiation contractile proteins.

In all further experiments additional evidence accumulated that irradiation primarily affects the cell membranes. So, for example, it is possible to imitate the radiation effect on muscle simply by increasing

the concentration of potassium ions in the outer medium. It had been shown in earlier experiments by other authors that the irreversible contraction of striated muscles of the frog observed *after* irradiation with very high doses is associated with a depolarization of the cell membrane, an increased loss of potassium from the cell, an increased concentration of sodium within the cells and with an increased oxygen consumption (Bergeder, 1955, 1958; Bergeder and Hockwin, 1960).

KCl belongs, together with acetylcholine, nicotine, conine and veratrine, to a group of drugs, which produce a reversible contraction of muscles accompanied by a loss of internal potassium and depolarization of the membrane. The effect of these substances can be neutralized if anodic polarization is applied to the muscle. Other drugs, such as chloroform, tribromethylene or lactic acid cause damage to the membrane and irreversible contractions (Fleckenstein, 1955). The reversible changes observed by us may be compared with the effect of the first mentioned group of drugs and only the "residual contraction" after high doses may be due to an irreversible damage of the membrane or other cell constituents.

Without going into further detail, I should like to present a model which I believe explains our results: in the resting muscle fibre irradiation produces an increased permeability which persists only during the course of irradiation and for a short time thereafter. The degree of the permeability change is a function of the dose-rate and its duration depends on the exposure time. During the time in which the permeability is changed a loss of potassium (and perhaps also of other ions) from the cells takes place and equivalent amounts of sodium enter the cell. In this way the quotient of the various internal to external electrolyte concentrations changes and this is accompanied by a fall of cell potentials and by contraction of the muscle fibres.

Shortly after irradiation the normal permeability state is almost completely restored. Due to metabolic processes ("sodium-pump") the original equilibrium of the electrolyte concentration is restored and the muscle relaxes. However, if the metabolic processes are inhibited the original electrolyte concentrations cannot be restored and therefore a certain degree of contraction persists. The most important question now is whether radiation attacks the cell membrane directly or indirectly by liberation or activation of pharmaco-active substances.

The considerably more sensitive reaction of nerve-muscle preparations of the same species to X-rays have not been investigated sufficiently for discussion of the underlying mechanism. One might assume though, that the nerve itself can be irritated by ionizing radiation and that in this case the site of action could be the cell membrane too.

Our observations of these immediate reactions of nerves and muscles of lower animals are in strong contrast to the well known radioresistance of isolated peripheral nerves and striated muscles of Amphibiae and mammals (Bergeder, 1955, 1958; Bergeder and Hockwin, 1960; Gerstner *et al.*, 1954; Darden, 1960). Only Bacq *et al.*, (1949) once reported that X-rays and  $\beta$ -rays of  $^{32}\text{P}$  produce immediate reversible contractions of the frog rectus.

One may conceive that this discrepancy is due to differences in methods, because the other authors usually recorded the effects a certain time *after* irradiation only. However, when we irradiated muscles and nerve-muscle preparations of the frog under the conditions described above no immediate reversible changes in tonus and motility could be registered. Only under very high dose-rates did the tonus rise slowly under and after irradiation, and the dose-effect-relationship is in agreement with those reported in the literature.

On the other hand, as mentioned already, various—if not all—smooth muscle organs of cold blooded animals and mammals react in a similar fashion to ionizing radiation as nerve-muscle preparations of lower animals. We have been able to confirm, in part, numerous earlier and long neglected observations (Swan, 1924) of radiation-induced changes in motion and tonus of smooth muscle organs under control of the autonomous nervous system, such as stomach, intestine, uterus, lung and blood vessels, and we observed that these effects depend on dose-rates comparable to those of the experiments described above. The elucidation of the differences in reaction of various types of muscles to ionizing radiation would contribute very much to our understanding of the action mechanism of radiation on the one side and of muscle physiology in general on the other. The immediate reactions of certain tissues and organs and their role in acute radiation effects on higher organism seem to deserve particular attention in future radiobiological studies. They may serve to call our attention more and more to the problems and methods of general physiology, which at times appears to have been neglected in radiobiological research.

It should be taken into consideration whether or not short-lived changes of permeability may occur not only in irradiated excitable cells such as nerves and muscles but also in other cell types. A few remarks made in this symposium by Alexander, Hollaender, Errera, Holmes and Passynsky can be taken as confirmations of the assumption, that some early radiation effects on various cells may be attributed to changes in their permeability.

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## DISCUSSION

SHABADASH: You have shown an immediate reaction of nerve-muscle preparation from leech or worm. What do you know about the more delayed effects, for example those occurring at five-minute intervals during the first hour when the muscles remain connected with the nerve chain?

What is the cause of failure in the experiments with frog preparations? In these experiments was the nervous apparatus left intact? I mean spinal gangliae and spinal cord.

HUG: We observed the preparations many hours after single or repeated irradiation and found no delayed effect after the onset of the immediate reactions. Even after 36 hr the reactivity to irradiation was preserved. For our comparative studies we used isolated preparations of frogs ischiaticus and gastrocnemius. We did not make *in vivo* studies with preserved spinal ganglia and spinal cord. Until now we have no explanation for the differences in reactivity between nerve-muscle preparations of lower animals and those of vertebrates. Perhaps species differences in the metabolism and nerve function are responsible.

MARCOVICH: Did you find any post-effects? Was it possible to observe immediate excitatory response to irradiation in the post-effect period?

HUG: Beside the "residual contraction" we observed no post-effect. Afterwards, except for high doses the response to other stimuli seems to be unchanged. These studies, however, are not yet finished.

LEBEDINSKY: Have you any experimental proofs indicating that the depolarization lies at the phenomenon basis of the radiation excitation? This is my view on the matter, but I would like to know whether you have any additional data beside those, which are already at our disposal?

HUG: For two years we have tried to discover immediate changes of the potential in muscle cells but failed to do so owing to technical difficulties.

TOBIAS: I have some data regarding reflex reactions of the mammal nerves and they are in good agreement with Dr. Hug's.

PASSYNSKY: Are the curves you have obtained exponential?

HUG: At a high dosage a quasi exponential curve of contraction was obtained. With lower dose rates more sigmoidal curves result (Fig. 5). It is very unlikely that the effects are produced by a one-hit mechanism.

POWERS: Did you observe any phenomena in the nerve-muscle preparation following irradiation of the nerve alone?

HUG: I did not irradiate isolated nerves without muscle.



# MECHANISMS OF CHEMICAL RADIATION PROTECTION

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## SUMMARY

The mechanisms by which added chemical substances—in particular cysteamine—alter the chemical changes produced by ionizing radiations are discussed. Examples are given of protection of proteins and nucleic acids by radical scavenging, energy transfer and repair. In mammals the protection given by —SH compounds is not due to the production of anoxia and is believed to be brought about by one or more of the chemical mechanisms working at the level of the primary lesion. Protection of mammals by histamine is due to the production of anoxia by pharmacological means because localized protection confined to the site of injection is not seen. When considering the mechanisms of protection every tissue or organ must be studied separately as several mechanisms may be active simultaneously or synergistically.

## INTRODUCTION

The term 'chemical protection' must be confined to those situations (discovered in 1949 by Bacq and Herve and Patt *et al.*, 1949) where the administration of a chemical *before* irradiation reduces the biological effects of a subsequent exposure to ionizing radiation. Post-irradiation treatments are not protection; they are therapy.

Chemical protection has been observed at all levels of radiobiology ranging from chemical changes of individual substances to death from radiation sickness in mammals. In every case, protection is much less marked with densely ionizing than with sparsely ionizing radiations. The uniform pattern of radiation protection and the fact that chemical protectors are inactive in wet systems (e.g. vegetative cells) when administered after (even very soon after) irradiation, indicate that these substances act by reducing the extent of the initial or primary chemical lesions.

Many reviews are available that give lists of the substances that protect, of the organisms that have been used and of various cells, tissues and functions that have been carefully studied in protected

living beings (Bacq and Alexander, 1961; Bacq, 1957, 1959; Patt, 1953, 1954; Pihl and Eldjarn, 1958; van Bekkum and Zaalberg, 1960).

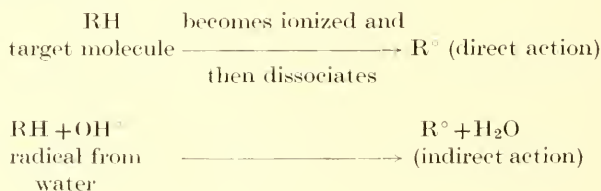
We shall limit our discussion not only to a few substances: cysteamine ( $\text{HS}-\text{CH}_2-\text{CH}_2-\text{NH}_2$  and some related compounds), but also to few systems (large molecules *in vitro* and mammals).

### PROTECTION AT THE CHEMICAL LEVEL

Polymers and proteins are quite suitable for determining the mechanisms of chemical protection because the conditions of the experiment (oxygen pressure, temperature, water content, concentration of the protector, quality of the radiation, etc.) can be drastically altered and accurately known, which is far from being the case with mammals.

Radiation damage to a given molecule can be prevented in one of three ways:

1. *By diverting the absorbed energy.* If the damage is being caused by indirect action then the protector intercepts the free radicals formed in the water and prevents them from reacting with the receptor. If the damage is due to direct action then the energy deposited in the macromolecule is transferred to the protector before it has time to cause chemical changes.
2. *By repairing the damaged molecule* (Alexander and Charlesby, 1955a). After becoming ionized, or reacting with the OH radicals formed by the ionization in the surrounding water, a molecule is not necessarily irreversibly damaged. A radical can be formed, with a lifetime in swollen systems of some  $10^{-5}$  sec, which can react with a protector in such a way as to be restored.  
For example,



In the absence of a protector  $\text{R}^\circ$  reacts further and becomes irreversibly damaged, while by combining with the protector (PH) it is restored or repaired.



Oxygen, on the other hand, tends to enhance radiation damage because it combines with  $\text{R}^\circ$  to give a radical  $\text{RO}_2^\circ$  which is no

longer capable of repair. The protective agent and oxygen are therefore working in opposition and in this way the fact that protection *in vivo* is more marked in aerobic than in anaerobic systems would be explained.

3. *By combining temporarily with the target molecule* and thereby rendering it more radio-resistant. This effect was first demonstrated by Dale (1942) who found that separately the prosthetic group and the protein part of an enzyme were more readily inactivated in dilute solution than the complete enzyme. Doherty (1952) found that chymotrypsin was less radiosensitive when combined with its substrate and several other examples of this type of protection have since been observed. Eldjarn and Pihl (1956) have suggested that the sulphhydryl-containing substances protect by reacting by an exchange process with the  $-S.S-$  groups, the destruction of which is assumed to constitute the primary lesion.

#### PROTECTION BY CYSTEAMINE IN MODEL SYSTEMS

In experiments with model substances cysteamine, which has been found very effective for protecting animals (Bacq *et al.*, 1951, 1953), has been shown to be capable of functioning by all of the mechanisms that have been discussed.

##### (a) *Competition for free radicals*

This has been demonstrated in several systems. Figure 1 shows its action in preventing radiation damage of bovine serum albumin in dilute solution. The amount of protein changed was determined from the sedimentation diagram measured in the ultracentrifuge (Rosen *et al.*, 1957). The rate of reaction of cysteamine with free radicals is much greater than that of the protein and almost all the cysteamine has to be destroyed before there is an effect on the protein (i.e. the dose-response curve has a threshold). However, even when the dose needed to change all the cysteamine has been exceeded, the rate of destruction of protein is still less than that in the unprotected solution. This is because the radiolysis product of cysteamine is still a weak protector. This is not the case with thiourea.

It is quite clear here that protection does not occur by a disulphide exchange process as postulated by Eldjarn and Pihl (1956) since in native albumin none of the seventeen disulphide bonds can be reduced by cysteamine because of steric inaccessibility. (Alexander and Hamilton, 1960).

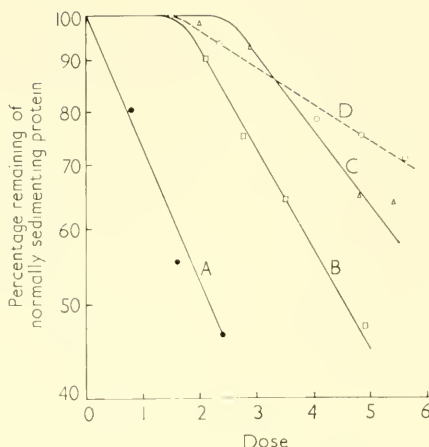


Fig. 1.—Protection of a 1 per cent solution of serum albumin by thiourea and by cysteamine against  $^{60}\text{Co}$   $\gamma$ -rays. Radiation damage of serum albumin was followed by changes in the sedimentation constant. The protective agents react very much more readily with OH radicals formed in the water so that the protein is unaffected until most of the protective agent has been destroyed.

Dose in  $r \times 10^5$

- A, No protecting agent;
- B,  $10^{-3}\text{M}$  thiourea;
- C,  $2 \times 10^{-3}\text{M}$  thiourea;
- D,  $2 \times 10^{-3}\text{M}$  cysteamine.

### (b) *Energy transfer*

The solid polymer polymethylmethacrylate is degraded by irradiation. The energy that has to be deposited in the polymer to produce a break in the polymer chain is 61 eV but if the polymer is cast as a film containing 10 per cent of cysteamine then 140 eV has to be supplied to achieve the same result (Alexander *et al.*, 1954). This protection results from the fact that more than half of the energy deposited in the polymer is transferred to the relatively small amount of cysteamine present. The possibility that this protection is due to a 'repair process' and not to energy transfer has been excluded (Alexander and Toms, 1958).

Gordy and Miyagawa (1960) demonstrated with electron spin resonance (ESR) protection by cysteamine against the direct effect of radiation in a protein. The number of free radicals, as given by the magnitude of the ESR signal, left after irradiating zein was much less if a small quantity of cysteamine was present. While an energy transfer mechanism seems the most probable explanation, the possibility that protection occurred by 'repair' or by disulphide exchange could not be excluded.

Libby *et al.*, (1961), made a similar observation with bovine serum

albumin which has been freeze-dried from a solution containing cysteamine. Figure 2 shows that the intensity of the ESR spectrum was much reduced by cysteamine (i.e. there were far fewer free radicals left

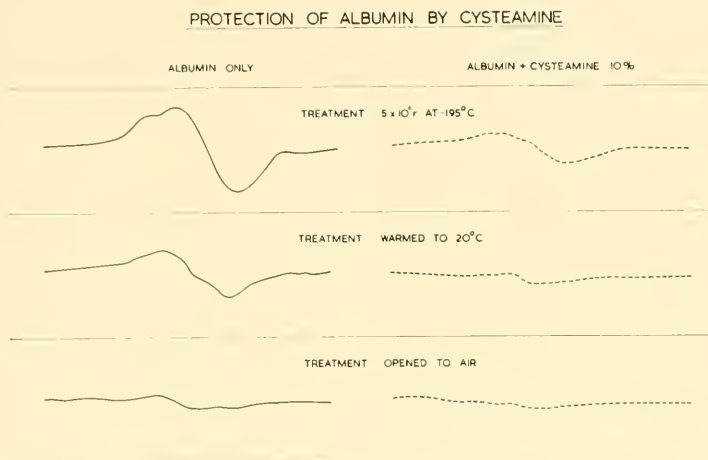


Fig. 2.—Protection of solid bovine serum albumin by cysteamine against the direct effect of  $^{60}\text{Co}$   $\gamma$ -rays as shown by the electron spin resonance pattern which measures the number of radicals formed. The protein-cysteamine mixture was prepared by freeze-drying a solution containing 10 parts of protein and 1 part of cysteamine.

in the protein). For this protein disulphide exchange can be excluded as the mechanism of protection since, as already mentioned, cysteamine does not react with the protein's disulphide groups under the conditions used.

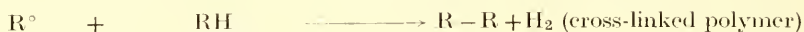
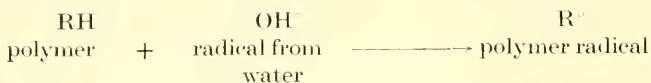
Of particular interest is the fact that if the protein is irradiated at the temperature of liquid nitrogen ( $-195^{\circ}\text{C}$ ) and its ESR spectrum measured at this temperature as well, many more free radicals are seen to be present than if the irradiation is done at room temperature. On allowing the protein to warm up the intensity of the ESR signal immediately decreases (i.e. the radicals disappear). Measurements at room temperature only reveal those radicals that are inherently stable and which are prevented from combining with one another by steric factors. The measurement at  $-195^{\circ}\text{C}$  reveals all the radicals that are formed, as at the low temperature subsequent chemical changes of the radicals are prevented. Cysteamine also reduces the number of radicals formed at  $-195^{\circ}\text{C}$  and this can only be due to energy transfer, and repair cannot be involved. Cysteamine appears very radiation-resistant as far as the formation of radicals is concerned, and when irradiated by itself gives only a very low ESR signal even at  $-195^{\circ}\text{C}$ . Thus, energy taken up by



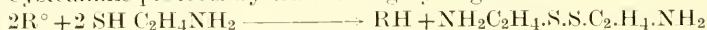
protein is prevented from forming a radical in the protein by transfer to the cysteamine in which it is dissipated without radical formation.

### (c) *Repair*

Cysteamine was shown to prevent radiation induced cross-linking of polyvinyl alcohol pyrrolidone in aqueous solutions (Alexander and Charlesby, 1955a, b).† The mechanism of cross-linking probably occurs as follows:



Cysteamine protects by transferring hydrogen to  $\text{R}^\cdot$



### *Protection of DNA against direct action‡*

Inactivation of bacteriophage by the direct action of X-rays is reduced in the presence of cysteine (Watson, 1952) or cysteamine (Marco-vich, 1958; Howard-Flanders, 1960), and this is presumably due to protection of DNA.

DNA becomes cross-linked by direct action if oxygen is excluded. Cysteamine protects against this process and it seems possible that the protection of phage is due to the prevention of cross-linking. Alexander and Stacey (1959) studied the effect of cysteamine on the cross-linking of DNA in the sperm or the isolated sperm heads from herring, salmon or trout. The sperm heads consist of a nucleoprotamine complex (65 per cent DNA and 35 per cent protamine) which is not swollen and contains less than 50 per cent water. On irradiation the DNA becomes cross-linked. This effect can be ascribed entirely to the direct action of radiation, and the free radicals formed in the water play no part. Table I shows that cysteamine reduces the amount of cross-linking. The mechanism of protection does not seem to be energy transfer since the

† In this system oxygen appears to protect when the polymer concentration is low by reacting to give a peroxide,



which cannot cross-link. This oxygen effect would not appear as protection in a biological system, since the molecule RH would lose its biological activity by peroxidation as well as by cross-linking.

‡ DNA can of course be protected against the indirect action of radiation by competitive removal of the radicals but the cysteamine concentration has to be high, as DNA reacts very readily with the radicals formed from water. Indeed DNA was shown (Alexander, 1953; Alexander *et al.*, 1955) to be an excellent protective agent against the indirect action of radiation on a polymer.

TABLE 1. *Protection against the direct action of  $5 \times 10^5$  rads of 2 MeV electrons on DNA in the heads of herring sperm.*†

Protective agent present in suspension (per cent)	DNA present as gel (per cent)
None	79
0.1 cysteamine	11
0.02 cysteamine	51
0.1 thiourea	66
0.02 thiourea	77
0.5 tyramine	54

On irradiation the DNA becomes cross-linked and is no longer soluble, but is present as a gel.

† The sperm heads were irradiated as a 12 per cent suspension in water. The sperm head nucleoprotein does not swell and is present as a hard sphere. Under these conditions the action of the radiation is entirely direct and free radicals formed in the water play no part.

ESR signal or irradiated sperm heads is not reduced at  $-195^\circ\text{C}$  by the addition of cysteamine. In this respect DNA behaves quite differently from serum albumin which has just been discussed. But, on warming to room temperature the radical concentration in the DNA containing cysteamine falls and direct evidence has now been obtained to show that this is due to "repair" of the DNA radicals by cysteamine (Ormerod and Alexander, 1961).

#### MECHANISMS OF PROTECTION OF LIVING SYSTEMS, MAINLY MAMMALS

The preceding examples have shown that the chemical reactivity of cysteamine is such that it is capable of protecting by several different mechanisms. Since cysteamine reduces radiation damage in a wide variety of biological systems it is possible that the same mechanisms are not involved in every case. For example the protection of dry seeds shown by Moutschen *et al.*, (1956) is paralleled by a reduction in the ESR signal of irradiated frozen yeast cells (Smaller and Avery, 1959) and an energy transfer mechanism seems to be indicated especially as very high doses are involved.

We believe that the Eldjarn and Pihl disulphide exchange hypothesis can be rejected as a mechanism of biological protection. At best it only provides for a reduction in radiation damage of proteins and these are unlikely to constitute an important primary lesion for reasons given in our other contribution to this colloquium. But even if damage to protein should prove to be important the data reported in this paper show that proteins are protected against both the direct, and indirect,

action of radiation by cysteamine under conditions when disulphide exchange cannot occur. No evidence has ever been produced why these other reactions are not the ones that protect proteins *in vivo*. The only reason for postulating that disulphide exchange is related to protection is that it is a reaction that occurs when cysteamine is injected into an animal, but recent research (Betz and Lelièvre, personal communication, 1960) indicates that there is no relation between the proportion of cysteamine bound in the tissues and the importance of protection.

The study of the mechanism of chemical protection in mammals is complicated by several factors: (a) Cysteamine injected into the vein or into the peritoneum is concentrated very unevenly by the tissues; at the time when the injected animal is irradiated, the protector is about 30 times more concentrated in bone-marrow, liver, spleen and intestine than in the testis (Eldjarn and Nygaard, 1954; Verly *et al.*, 1954; Verly 1955; Nelson and Ullberg, 1960). A large part of the activity of cysteamine is probably due to the fact that it penetrates rapidly within the cells of some critical organs (bone-marrow, liver, spleen, intestine, hypophysis. (b) The oxygen pressure in a normal man is high in the skin, muscles and spleen, but low in the bone-marrow; in this last tissue the oxygen tension is not increased (as it is in other tissues) by breathing pure oxygen (Cater and Silver, 1960). Thus the oxygen effect must be small in the bone-marrow although this tissue is known to be very well protected in rodents by cysteamine and related substances.

Every tissue or organ within the mammal must be studied separately. Several mechanisms may be active simultaneously and synergistically.

Many authors (see for instance Gray *et al.*, 1952a, b) have suggested that some protectors functioned by causing, via a pharmacological pathway, the depletion of tissue oxygen. L. H. Gray (1960) also subscribed to this view and suggested that the sulphhydryl compounds remove oxygen by direct combination due to autoxidation which occurs relatively rapidly under physiological conditions.

Several methods may be used to exclude anoxia as the principal mechanism of chemical protection in mammals.

(a) The use of cysteamine (the  $-S-S-$  oxidized compound related to cysteamine) avoids the criticism of Gray and Scott. Many protective effects of cysteamine have been duplicated with cystamine—but not all of them. Careful study of the difference in tissue fixation, metabolism, anoxiating effect, quality and intensity of protection, etc., between the SH compound (cysteamine) and the  $S-S$  derivative (cystamine) should be undertaken.

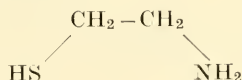
(b) If the injected protector induces within the tissues a decreased

oxygen pressure (as measured by electrodes introduced in the fluid surrounding the cells)†, if the time course of tissue anoxia is the same as that of protection, then the possibility exists that anoxia is involved as the mode of action of the protector. This is the case for histamine, but not for cysteamine (Van der Meer and van Bakkum, 1959; van der Meer *et al.*, 1959). An antihistamine drug, Phenergan, which abolishes the hypotension and decreases the fall in oxygen tension in the spleen after histamine injection, also decreases the radio-protective effect of the amine. Thus the action of histamine seems to depend on a physiological, not a physico-chemical mechanism. Careful measurements of oxygen tension in tissues have now been made in a number of laboratories (e.g. Grayevsky in this symposium and van der Meer *et al.*, 1961) and there is complete agreement that SH-protectors do not give rise to a significant reduction in oxygen levels and that, there, radio-protective action cannot be explained by the anoxia hypothesis.

(c) If a substance protects isolated mammalian cells (thymocytes or reticulocytes) irradiated *in vitro* when the oxygen pressure does not change, then anoxia is not involved; some mechanism or mechanisms involving free radicals must be at work, which are also probably active in the protection of the whole mammal. Such is the case for cysteamine and cystamine (Nizet *et al.*, 1952; Betz and Booz, 1957; van der Meer and van Bakkum, 1959). On the contrary histamine does not seem to protect isolated mammalian cells *in vitro* (van der Meer *et al.*, 1959; van der Meer and van Bakkum, 1959), although it has some activity in protecting polymethacrylate in aqueous solution (Alexander, 1953; Alexander *et al.*, 1955).

(d) At least one test in mammals (extremely rapid increased permeability of connective skin membranes in rat and man) has been shown to be independent of oxygen and to respond very well to cysteamine and other protectors (Brinkman and Lamberts, 1960; Bacq *et al.*, 1959).‡

(e) Several papers have given useful information on the relation between chemical structure and protective power against ionizing radiation in mammals (Bacq and Alexander, 1961; Alexander, 1953; Alexander *et al.*, 1955; Doherty *et al.*, 1957) in the series of cysteamine. The conclusions are the following



† Anoxia is the consequence of a slowing down of blood circulation and decreased oxygen saturation of capillary blood.

‡ According to Brinkman, oxygen decreases this effect of X-rays.



(1) substitution of the H in the S or N decreases the activity; in other words the sulphydryl and amino functions must be free. Activity is retained if one hydrogen of the amino group is substituted by a guanidyl group to give mercaptoethylguanidine (Doherty *et al.*, 1957). This substitution does not change greatly the physico-chemical properties of the molecule, the guanidine being a strong basic group.

(2) if the length of the carbon chain is increased beyond 3 C atoms, the activity falls sharply; in other words, there is an optimal distance of 2 or 3 C between a strong covalent group (SH) and a basic group (NH<sub>2</sub>).

The Oak Ridge group has perfectly understood that these facts cannot be explained by anoxia or the mixed disulphide theory, but are readily interpreted by mechanisms involving free radicals. As a matter of fact the views of this group are very close to ours.

The structure which favours chelation, also favours chemical protection (Alexander *et al.*, 1955).

(f) The phenomenon of local protection in mammals is also important because general effects on circulation causing anoxia are absent. For instance the vaginal or the rectal epithelium is protected by local application of cysteamine or cystamine (for bibliography, see Bacq and Alexander, 1955). The skin of man, rat and mice is protected locally by cysteine or cysteamine injected subcutaneously in small amounts or introduced in the skin by ionophoresis. Here again the difference between cysteamine and histamine is obvious (Radivojevitch *et al.*, 1960):

(i) When very small amounts (25  $\mu$ g) of cysteamine are injected subcutaneously into 8 days old C 57 black mice, the hair falls after irradiation except around the injected spot; large doses do not succeed in protecting the whole skin.

(ii) Histamine does not protect locally, but if enough histamine is injected subcutaneously, *the whole skin* is protected apparently because the reduced blood pressure and slow circulation have induced a sufficiently deep anoxia.

Thus, as far as cysteamine in mammals is concerned, it seems that neither the mixed disulphide hypothesis, nor anoxia can explain the observed facts. We believe that one or several mechanisms involving free radicals are much more likely to be responsible for the protective ability of cysteamine in mammals.

Protection by competitive removal of free radicals and by repair is possible in addition to direct energy transfer as observed in model systems. The repair mechanism is likely to play an important part, since it provides an explanation why protection is more marked in aerated cells. Once the radiosensitivity has been reduced by anoxia, chemical protection is in general much less marked. In 1955 we tenta-





Fig. 3.—All mice are 14 days old. From left to right: 1, Normal mouse, C57 Black; 2, Mouse irradiated on whole body with 550 r X-rays when 8 days old; 3, Mouse similarly irradiated except that a *subcutaneous* injection of 0.75 mg of cysteamine has been given 5 min before irradiation at the posterior region of the back; 4, Mouse similarly irradiated but after a *subcutaneous* injection of 1.1 mg of histamine in the same region.

Local protection is obvious in mouse 3, general protection in mouse 4.

For more information see Bacq, Beaumariage and Radivojevitich (*Bull. Acad. Méd. Belge*, 1961, VII, ser. 1, 519).

tively proposed that this behaviour could be explained either by the  $\text{HO}_2$  radical hypothesis (Alexander, 1953; Alexander *et al.*, 1955) or by reaction with an organic radical (Alexander and Charlesby, 1955a) in which repair by the protector and irreversible damage by oxygen addition could be considered as opposing processes. Possibly in the absence of oxygen, the radical  $\text{R}^\circ$  may undergo spontaneous repair by other cell constituents, and added chemicals do not then protect. In the presence of oxygen the radical  $\text{R}^\circ$  becomes peroxidized unless there is a protective agent present which prevents this reaction and thereby protects against that part of radiation injury that requires oxygen.

In the last five years chemical investigations (cf. Bacq and Alexander, 1955) have made it less likely that  $\text{HO}_2$  radicals (formed in water by  $\text{H} + \text{O}_2$ ) are chiefly responsible for cell damage and this is why we have shifted our emphasis to the repair mechanism. However, either process requires that cysteamine protects as a result of reacting with a free radical.

#### ACKNOWLEDGMENTS

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## DISCUSSION

POWERS: While discussing the effect of cysteamine on the ESR, observed in albumin following irradiation, you have said that it should be connected apparently with the energy transfer and not with the decrease in the number of radicals caused by the transfer of the H-atom from the protector. In this connection I have two questions to ask.

1. What do you mean by "energy transfer"?
2. How do you distinguish it from the reaction of the radicals with the H-atom by means of the ESR spectrum?

ALEXANDER: We believe that cysteamine does not protect albumin by a repair process because the number of radicals formed (as measured with ESR) is reduced even at  $-195^{\circ}\text{C}$  when the bimolecular reaction involved for repair by H transfer cannot occur.

As to the expression "energy transfer", these words are used when it is not known why the molecule does not change under some influence. There are many forms of "energy transfer" of this kind.

POWERS: As regards cysteamine activity at low temperatures it seems to me that the data on the activation energy, available in the literature, indicate that the

migration occurs in the solid compounds and suggests that cysteamine at these temperatures also is acting as a hydrogen donator and reacts with the radicals.

ALEXANDER: We rule out this possibility on the grounds not so much of dynamic, as of kinetic, considerations. At such low temperatures cysteamine cannot reach the place where it is protecting.

SHABADASH: I would like to know Prof. Bacq's opinion regarding disagreements between his and Prof. Arbuzov's (Leningrad) data on the quantity of sulfhydryl protectors in animal organs. According to Arbuzov's data 1 to 4 hr following administration of  $^{35}\text{S}$ -labelled cysteamine the highest cysteamine concentration is observed in the central nervous system.

What are the quantitative data obtained in your laboratory on the cysteamine content of the brain, especially of the subcortical structures and the hypothalamic region?

BACQ: What I was talking about was a very short review of three different investigations. Some of the experiments had been carried out by us, some performed by other authors. Recently in Sweden the topography of  $^{35}\text{S}$  distribution has been studied following administration of the  $^{35}\text{S}$ -labelled cysteamine to a mouse. We analysed the brain as a whole, and not its particular areas. As to the Swedish investigation, in that work no specific concentration of the substance in particular areas of the brain was found.

The difficulty is that while assaying  $^{35}\text{S}$  we should be sure that it is still connected with the cysteamine molecule; this molecule breaks down very rapidly while in the body.

I have no explanations for the discrepancies you referred to.

GRAY: I fully share Prof. Bacq's view. It seems to me that the protection given by the majority of the active agents may be connected with anoxia. Nevertheless maximum protection due to anoxia is slightly less than that produced by pharmacological agents. We should adopt a highly critical attitude. It would be better to experiment on cell suspensions at known oxygen pressure levels. Under these conditions we could study more effectively the metabolic changes occurring when protective agents are used.

KALMANSON: What is the absolute sensitivity of the equipment? What is the minimum quantity of the radicals you can still record? What are the free radical concentration values of the curves you have shown? What are the absolute changes of free radical concentration in your experiments?

ALEXANDER: At the sensitivity used  $7 \times 10^{14}$  radicals could be detected in a 100 mg sample. In most of the experiments the concentration of radicals was of radicals of the order of  $10^{17}$ . We detected no free radicals in unirradiated protein and the effect of radiation was therefore to introduce about  $10^{17}$  radicals in 100 mg and this requires doses of the order of several megarads.



# KINETICS OF PRIMARY REACTIONS AND CHEMICAL PROTECTION

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## SUMMARY

The kinetics of the protective reaction of anti-oxidizing agents was studied in order to analyse the mechanisms of the primary reactions. New facts have been adduced which testify to the chain mechanism of radiochemical oxidizing reactions induced by radiation. It has been established that chemical protection is afforded not only when the oxygen level is reduced but also when the pressure is raised above a certain critical level which is the characteristic property of oxidizing reactions of the chain type, with branched chains.

It has been established that the kinetics of the variation of the amount of lipoperoxides in the tissues of irradiated organisms corresponds to the formula for the accumulation of intermediate products of branched chain reactions. But the kinetics for the variation in unsaturated fatty acids is analogous to the kinetics for the accumulation of the final products in branched chain reactions.

The presence of chain oxidizing reactions is confirmed by the discovery of very weak luminescence in the region of 4,000 Å to 5,200 Å which increases considerably under irradiation conditions.

The quantitative analysis of the data concerning the protective action of increased oxygen and of anti-oxidants has shown that these factors are inhibitors of oxidative chain reactions in cell lipids. It has also been shown that the action of anti-oxidants is restricted and in the most favourable conditions they cannot absorb more than 50 per cent of the dose. This incomplete protection shows that the oxidation chain reactions are not the sole primary reactions and apart from them there arise, and are developed, non-oxidative reactions in various biological substrata. Investigations have shown that this is mainly a reaction of an autolytic character which develops after irradiation. The protective substances which contain —SH groups activate this type of reaction. The investigations were carried out in yeast of haploid and diploid strains, in mice and in rats.

Ionizing radiations, with strong chemical action, lead to the formation of radicals and ions. An essential part is played by those that can induce primary radiochemical reactions developing after irradiation, and involving a great number of molecules per single ionizing event.

It is very difficult to establish the nature of primary chemical reactions on the basis of chemical changes in cells and tissues alone, as the concentration of the products of primary reactions is very low and lies



outside the scope of analytical methods. Moreover, secondary reactions begin to develop very rapidly and it is very difficult to differentiate the former from the latter.

At the same time the kinetic peculiarities of primary radiochemical reactions in various components of cells and tissues leave a natural imprint on the development of secondary reactions and on the biological manifestation of damage from radiation. This temporal dependence helps to determine the character of the basic primary processes.

The action of protective chemical agents is clearly directed towards these primary reactions. These agents inhibit them. Therefore the study of kinetics can provide us with valuable information concerning the nature of primary processes. The most peculiar feature in radiation damage is the so-called oxygen effect, a lowered radiosensitivity of irradiated organisms at low oxygen pressures.

This effect, described by many authors for various organisms, has been interpreted as a result of the decrease in the products of water radiolysis and especially of the radical  $\text{HO}_2$ . In order to understand the mechanism of the reactions that are inhibited at low oxygen pressure, one must pay attention to the kinetic peculiarities of this phenomenon. The peculiar kinetic feature of the protective action of oxygen, when its pressure is lowered, consists in the fact that the protective effect does not increase gradually with the lowering of oxygen pressure, but goes up rapidly after a certain pressure threshold. Therefore the lethal curves (Sumarukov, 1958) depending on oxygen pressure have always the shape of parametric parabolas. (Fig. 1).

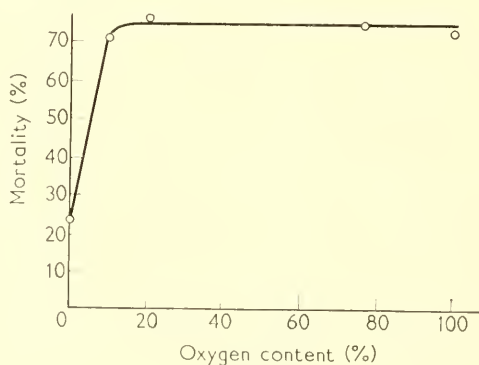


Fig. 1.

This kinetic peculiarity has been established by many investigators who have studied the phenomenon quantitatively on mice, tissue (sarcoma cells) bacteria, bean roots and others.

With the lowering of oxygen pressure in water irradiation, the amount of the products of water radiolysis decreases. However the kinetic curves of this process are evidently different. In this case the changes in the oxidizing capacity of water give a sloping curve.

The dependence from oxygen pressure of the threshold of the rate of the reaction has been known in chemical kinetics and is a peculiar feature of oxidizing chain reactions with branching chains. This type is usual in the oxidation of lipids, as lipoperoxide compounds easily arise, securing the branching of the reactions. This type of reaction is, according to Semenov (1958), characterized by anomalous dependence of the rate of oxidation from oxygen pressure. The breaking of chains and the inhibition of the reactions is achieved both at low and high pressures. Independently of the radiation effect the rise in oxygen pressure has a toxic effect on organisms. Against the background of this toxic effect it is very difficult to establish the influence of increased oxygen pressure on the primary reactions.

But that is possible for organisms for which oxygen is not toxic. In our laboratory we have undertaken the study of the effect of oxygen with a very wide range of pressures from 0 to 11 atm on a yeast which can survive in pure oxygen at any pressure.

Studying the relationship of survival after radiation with oxygen pressure, Kolontarov (1958, 1959) has discovered that not only a decrease but an increase of oxygen pressure as well has a protective effect (Fig. 2).

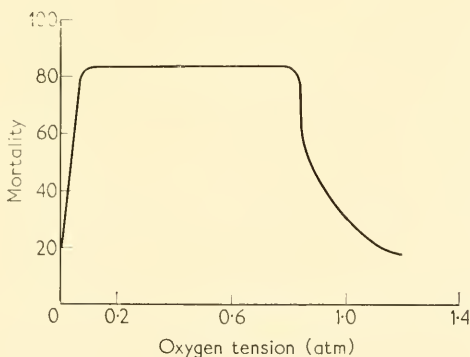


Fig. 2.

In no connection with our work, a paper has recently been published by Alper (1959) in which it is stated that irradiation of the yeast *Saccharomyces vini* in an atmosphere of pure oxygen increases its capacity

for survival. In higher animals this effect is masked due to the toxic effect. Nevertheless, Zhuravlev (1959) in our laboratory has shown on much material that irradiation of mice does not increase the toxic effect of oxygen at pressures close to one atmosphere, and a slight increase of the ability to survive has been noted. The curve in this case may be regarded as a sum total of the toxic and the protective effects (Fig. 3).

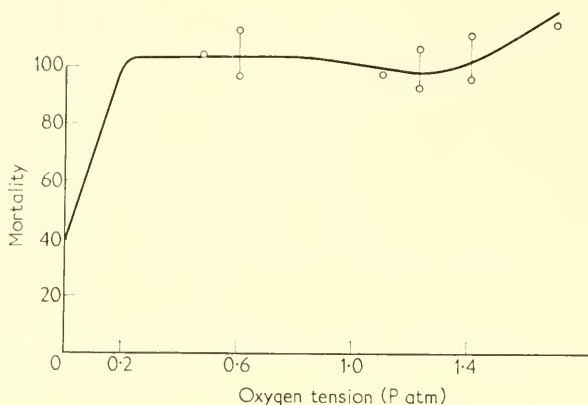


Fig. 3.

The protection of yeast at high oxygen pressures reveals the nature of at least one of the primary reactions. If protection at low oxygen pressure can be explained by the decrease in the products of water radiolysis (if we ignore the peculiar features of the kinetics of the process) it is difficult to connect with it protection at increased pressure. With an increase of oxygen pressure the amount of the products of water radiolysis, especially of oxidizing products, must increase to a threshold saturation at high pressures.

On the basis of kinetic data we should suppose that the kinetic threshold of oxygen protection is determined by an inhibition of the oxidizing reaction developing in the organic lipid structural phases of cells (Zhuravlev, 1959).

Experiments with various biogenic components show that this type of reaction could hardly arise on a different substrate. In that connection we have undertaken a wide investigation of protective action in two lines of yeast, haploid and diploid, at high oxygen pressures. These two lines greatly differ in radiosensitivity, 3,000 r for haploid LD<sub>50</sub> and 40,000 r for diploid LD<sub>50</sub>.

It has been established that both these lines behave differently at

high oxygen pressures during irradiation. Protective action in the diploid was much weaker than in the haploid when the yeast was irradiated in water suspensions (Fig. 4).

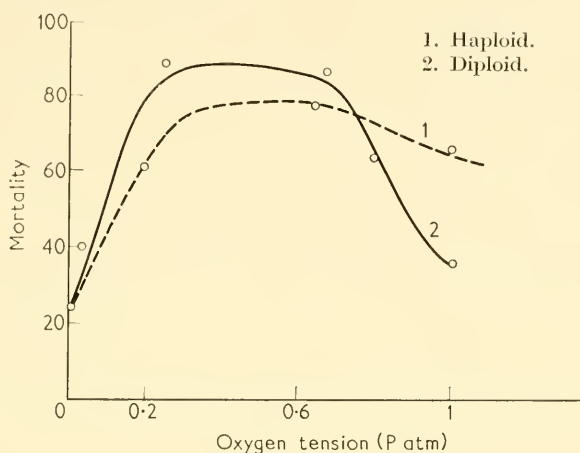


Fig. 4.

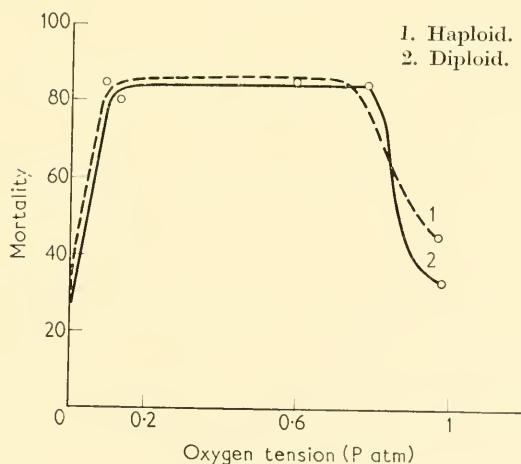


Fig. 5.

During irradiation of agar cultures the protective effect increases in the diploid, reaching that of the haploid. Protective action in the haploid, in agar cultures, is the same as in suspensions (Fig. 5).

This can be explained as follows. The high resistance of the diploid line, as compared to the haploid, shows that induced primary radiation reactions developed more weakly in diploid cells and had a lower ionic

yield. The differences in radiosensitivity by irradiation in oxygen are greatest in agar cultures (in the atmosphere) where the  $LD_{50}$  is of the order of 100 to 160,000 r for the diploid and 4,000 r for the haploid.

For irradiation in water suspensions in oxygen it is 40,000 r for the diploid  $LD_{50}$ , and 4,000 r for the haploid. It shows that, in suspensions of diploid cells, an indirect effect, through the products of water radiolysis, prevails, on which an increase in oxygen pressure has no effect.

In irradiation of agar cultures a part is played by the reactions indirectly induced in the organic phases of the cells. In haploid cells the physico-chemical conditions are such that reactions in organic phases are easily induced by direct irradiation and the increase of water in the external medium cannot increase the lethal effect. This reflected in the lethality curves at lower oxygen pressure in water suspensions. From that point of view one should analyse the primary reactions in multicellular organisms. As has been pointed out the lethality curves in connection with oxygen pressure have a marked threshold character in mammals, insects, etc. (Fig. 1).

This makes it possible to conclude that primary, oxidizing radiochemical reactions arise and develop in the main in the organic phases of the cells and this fact apparently determines high radiosensitivity.

The considerations given above are confirmed by data, constantly published, which establish the appearance of active lipoperoxides in irradiated organisms (Morgan and Philpott, 1954).

It has been demonstrated that the formation of these peroxides can be discovered during irradiation and that it is hindered by protective substances.

We have studied kinetic regularities in the formation of lipoperoxides in the liver of rats after various doses of irradiation. Malts (1960) has shown that during irradiation three chain reactions in the lipids of the liver with different kinetic parameters take place. The changes in the amount of peroxides correspond to the kinetic accumulation of intermediate products of chain reactions, i. e. the amount of peroxide is determined by the rate of the reaction at the given moment (Fig. 6).

There are grounds for the assumption that these reactions in biolipids are connected with the destruction of anti-oxidants always present there. We have established that, after irradiation, there is a gradual decrease of anti-peroxidants in the lipids of the liver of rats and mice (Zhuravlev, 1960; Malts, 1960) parallel to the reaction of radiation oxidation.

As we know, oxidizing reactions are considerably slower in the presence of anti-oxidants, though not completely stopped. A number of investigations have shown that a slight decrease of the anti-oxidant



level (5 to 10 per cent) is sufficient to increase a slow reaction. Apparently the same applies to irradiated biolipids.

We have established for instance (Tarusov, 1957; Polivoda, 1960; Zhuravlev, 1960) that lipid extracts from the livers of irradiated mice are less able to decrease chemoluminescence, observed in oleic acid during its oxidation in the atmosphere, than extracts from control

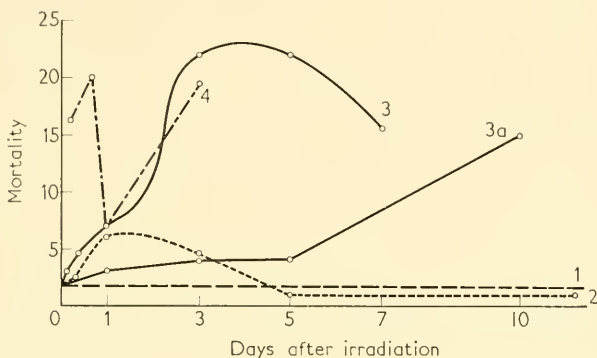


Fig. 6.

animals. With the use of highly sensitive methods for the detection of weak radiation we could establish that homogenates of the liver of mice were producing slight radiation of the type observed in the oxidation of fats, (about 4,000 to 5,000 r). In irradiated animals it is more intensive. Cysteine and other protective substances stop this luminescence.

The second important feature is that with an increase of the density of ionization the main kinetic indicators of the radiochemical oxidizing reaction are weakened and even disappear; the oxygen effect is lacking, and anti-oxidizing substances do not give protection. In the literature there are very few data on the absolute value of chemical protection, i.e. the elimination dose. Therefore we undertook corresponding quantitative investigations on yeast and mice and evaluated the data in the literature. Each agent was tested in a series of experiments under optimal conditions for the manifestation of protective action ( $\gamma$ -radiation). It has been established that under optimal conditions chemical protection neutralized less than 50 per cent of the dose. Maximum protection came from the oxygen effect, both at an increase and a decrease of oxygen pressure (for yeast). That has been earlier noted by some authors.

The data show that besides primary oxidizing reactions there exist

reactions of a different kind. Radiochemical investigations have shown that oxidizing reactions occur mostly in biogenic compounds, especially in water solution, but some investigations show that even under these conditions there develop non-oxidizing reactions with a high ionic yield, not affected by oxygen and protective substances. A limited protective action of the inhibitors with anti-oxidizing properties is due, perhaps, to the interaction of two, probably independent reactions of an oxidizing and non-oxidizing character, developing at different rates on different substrates. At present the best criterion of protective action is the survival of the organisms. It can be produced by various primary and initial reactions.

The prevailing role is played by those that can most rapidly lead to such secondary changes that bring about a lethal outcome. If we want to draw a scheme of two independent reactions we have: reaction A, a non-oxidizing, but rapid reaction, with a low ionic yield; and reaction B, oxidizing in its character, with a higher ionic yield, but slow, with a considerable incubation period. The task is to establish the statistical law of the lethal outcome of these two reactions in connection with the régime of irradiation. With minimal doses and no density of ionization it is more probable that reaction B will bring about the lethal outcome at a later date and a considerable number of organisms will pass through the earlier reaction A which will produce fewer chemical changes below the critical threshold. At a higher density of ionization reaction A will be more rapid and will cause damage bringing about the lethal outcome.

At present not enough data on non-oxidizing reactions playing a part in primary processes are available, as these reactions are masked by other processes. However, one group of the so-called autolytic reactions, whose nature has been insufficiently studied has attracted attention.

It has been established (Benevolensky, 1960) that homogenates of the liver of irradiated rats, already in the early stages are able to split unsaturated fatty acids with a non-haemolytic action from a centrifugate of the liver inactivated by heating.

This does not apply to homogenates of non-irradiated animals, but the reaction occurred if the liver homogenates of normal animals subjected to autolysis were added.

It has been also established (Burlakova, 1960) that already in the early stages the tissues of irradiated mice, whose electric properties did not differ from those of normal animals, with time fail to polarize the electric current, as opposed to control animals in which this process is slow. In irradiated animals autolysis is slower than in normal. It has

been confirmed by direct chemical determination of nitrogen content and it has also been established that autolysis is more energetic in irradiated animals than in control ones and that their blood cannot inhibit the autolytic process (Mkartychan, 1960).

It is very important that protective agents (lack of oxygen, cysteine) activate the autolytic processes. It has been, for instance, established that cysteine and mercaptoethylamine activate autolysis, and speed up radiation damage if they are systematically injected after irradiation, in the case of internal damage caused by radioactive substances (cerium, strontium) and by external radiation (Mkartychan, 1960). One could therefore suppose that a lack of protective action and even a negative effect of protective agents is due to the activation of the autolytic process. This must be considered as the first step in the understanding of the kinetics of primary reactions.

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#### DISCUSSION

PARIBOK: What was the experimental procedure: was oxygen under pressure used or compressed air?

TARUSOV: In case of extreme variations it was pure oxygen, while in other experiments a mixture was used.

PARIBOK: I should like to draw attention to one consideration. You have mentioned difficulties involved in work with oxygen under pressure when experimenting with mammals, due to the oxygen's toxicity. Actually many are well acquainted with this difficulty, but symptoms appear only above 2 atm pressure,

whereas according to your data on yeast, protection occurred even at a pressure of less than 1 atm.

I experimented on animals. Cats survive oxygen pressures amounting even to 3.5 to 4.0 atm. It opens the way to testing the protective effect of oxygen on large animals.

TARUSOV: Our conditions do not allow us to use animals weighing more than 20 to 30 g; it is our limit.

MARCOVICH: How do you irradiate yeast in agar culture?

TARUSOV: The agar plate is placed within a cylinder, from which the air is evacuated and replaced by the gas mixture. Then the system is transferred into the irradiation chamber.

MARCOVICH: What is the procedure for transferring yeasts to agar and what is their condition on agar?

TARUSOV: Special vessels with an agar layer are inoculated with yeast. Yeast spreads over this layer forming a film of practically uniform thickness.

KUZIN: What data do you have at your disposal to warrant the view that the appearance of lipid peroxides you have demonstrated three days after the exposure is due to a chemical chain reaction and not to profound metabolic disturbances in the lipids which develop at this period in the liver?

TARUSOV: Regardless of anything else, the appearance of peroxides is not the main index here. I regard it as a result. As I have said, secondary reactions are the reflection of the primary ones. In our opinion the basic process is connected not with these peroxides, but with anti-oxidants, which are released as a result of irradiation; release of the anti-oxidants opens the way to oxidation, with no disturbance in the general metabolism.

TUMMERMAN: In connection with your very interesting observation that oxidation of lipids gives rise to a visible luminescence I would like to say that it is not a specific peculiarity of the lipids. We have observed similar luminescence in visible regions of the spectrum in the case of numerous proteins, some amino acids and carbohydrates. Even ordinary paper placed in the dark before a sufficiently sensitive receiver and heated to about 150°C emits visible light of sufficient intensity, probably with two maxima—one in the green, and another in the red region of the spectrum—when no irradiation has been applied. This effect is highly dependent on the presence of oxygen and in general conforms to the same kinetic laws. At oxygen pressures up to 10 cm the effect does not disappear, but it disappears completely at 0.1 cm pressure.

Do you not think, that such luminescence accompanying oxidation of very different biological substrates, may be related to those very interesting phenomena discovered by Professor Mitchell which were mentioned in Dr. Holmes' report?

TARUSOV: Of course, there is no question of heating the animals to 150°. The luminescence described occurs in normal animals also, but it has only a small intensity; it is activated by irradiation.

The difference from your data, according to your report, consists in the fact that the spectrum breaks off in the red region, and there is no maximum. We do

not have a two-peak curve, as is the case in your experiments, whereas the maximum in the green region is identical to that found in the case of visible bioluminescence. In other words our data differ from the phenomenon you have described.

TOBIAS: When chain reactions are being studied it often happens that the initiation of the reactions depends on the radiation intensity. Did you find any dependence on intensity?

TARUSOV: In our experiments the main parameter was the time. Radiation was almost always the same, i.e. in most cases we did not vary either the dose or the radiation intensity. We tried to work in the region of low radiation density. Under these conditions protective effects manifest themselves more readily.

BACQ: I also studied peroxides following irradiation and found that the appearance of peroxides is not an early, but an immediate, reaction. Another important point is that in animals there is a natural anti-oxidant,  $\alpha$ -tocopherol. When you take a fasting animal, the fat disappears, but  $\alpha$ -tocopherol does not disappear. Thus tissue is living under conditions of increased  $\alpha$ -tocopherol content, and this should be borne in mind.

TARUSOV: We have also found that peroxides appear immediately. In the work of Romanzev (who is present here), it was established that peroxides and lipoperoxides make their appearance during the irradiation. But I guarantee that there are no analytical methods which would enable us to discover them at the levels of the irradiation present. And it is a proof that at once, already at the first stage, the chain reactions occur. By the way, Romanzev has shown that this initial accumulation of the peroxides is inhibited by sulphhydryl protective compounds.

PASSYNSKY: Did you study whether the oxidation chain-reactions occur in two-layer films of the lipids?

TARUSOV: Of course there are in the body two-layer films of lipids, but there are also films in which these two layers are lacking; not all is distributed in two layers.

PASSYNSKY: Whatever the chain reaction is it may occur only when a certain critical mass is present. What is the critical mass for the oxidation of lipids?

TARUSOV: We did not determine the critical mass.

PASSYNSKY: I think that determination of the critical mass is quite indispensable if the existence of chain reactions for lipid oxidation is to be accepted.

TARUSOV: I do not believe that these concepts can yet be applied to the cell.





## GENERAL DISCUSSION

BACQ: Ladies and gentlemen, in order to make our General Discussion fruitful, and to guide it into the channel of the most pressing problems, I wish to put before you a list of questions which follow logically from all the material discussed at the symposium and around which, in my opinion, it is advantageous to open the discussion. These questions are as follows.

I. Electron spin resonance spectra show that free radicals are important in the primary mechanisms of radiation action. Using this method one can obtain an experimental approximation to the investigation of excited states.

II. Mitosis is more radiosensitive than DNA synthesis. These two processes do not have to be interconnected. Does anyone have any ideas as to the mechanisms that regulate the rate of mitosis?

III. Many primary lesions are reparable, even injuries to the nuclear substance. Do these restorations occur as a result of natural metabolic processes or by artificial action?

IV. Chromosome breaks do not only occur as the result of direct action, they must be regarded as secondary effects which are greatly modified by the metabolic activity of the cell.

V. There is not just one primary chemical injury caused by ionizing radiation, but a whole series of changes which are most properly regarded as injuries to the structure (membranes, intracellular structures), which destroy the normal forms of contact inside the cell between enzymes and substrates—the cellular auto-regulation of metabolic processes.

VI. Is the oxygen effect universal? It occurs in primary chemical reactions. Part of the damage caused by ionizing radiation does not depend upon oxygen.

VII. The chemical protective substances have diverse action mechanisms which can be simultaneous or synergistic. Each protective system must be considered separately. One cannot draw simple conclusions.

VIII. Some elementary physiological systems are radiosensitive, some are very resistant. How can one explain this difference?

If those present have no objection to the list I have proposed and also no objections to allowing not only the members of the symposium, but

also certain guests who are present here to make brief comments and to speak, we shall proceed to the discussion.

I. Study of electron spin resonance spectra has shown that the free radicals which are formed at the instant of radiation play a large part in radiation damage. Is it possible to investigate the excited states experimentally?

PASSYNSKY: In regard to the question of electron spin resonance, I wish only to caution against an excessively wide interpretation of the data.

It is possible that the measured effect comprises only a small part of the primary changes, and therefore the method must be developed and measurements taken at the moment of irradiation. In radiation conditions many excited molecules are produced without any re-arrangement of the spin or without detachment of an electron; for example with the absorption of 3 to 5 eV. Such molecules are capable of many new reactions. I have, in the past, put forward the concept of the basic role of the excitation of molecules of protein under radiation conditions. Blumenfeld, whose work on ESR I value highly, said in reply to my question that, in his opinion, the total molecular energy levels for the "conductivity channels" are not in general filled in the native molecules of protein and DNA, and can only be used in enzyme catalysis and irradiation. I agree with him in regard to the irradiation (by ionizing radiations or by u.v. rays), but I do not agree with him on the question of enzyme catalysis. The excitation energy of the protein amounts to about 2.5 eV and the energy of excitation of all the enzymatic reactions to approximately 0.4 to 0.7 eV. This question has been specifically examined by Lowry† and his final conclusion is as follows:

Enzymes work only in the ground electron state. It is evident that in the normal life of the organism, semi-conductor-like chain reactions do not take place. Under the influence of radiation a transition to an excited state is possible, which must be examined without assuming a simple change in ESR spectra. This important method represents merely one of the methods of investigation and must be supported by many others. I think that the measurement of the quenching at low temperatures is very important, provided that chemiluminescence, especially of an oxidizing nature, is excluded.

† In "The Enzymes", Vol. I, (Paul D. Boyer, Henry Lardy and Karl Myrback, eds.) 2nd edition, 1959. Academic Press, New York and London.

BLUMENFELD: I entirely agree with Passynsky that the excited electron states do not play any part in the basic, normal biochemical processes. But the fact is that what we study by the methods of electron spin resonance when enzyme processes are taking place is a ground state and not an excited state. This demands a special discussion, and I will not dwell any longer on this matter.

In regard to the question of the possibility of utilizing the ESR method in radiobiology, it is necessary to bear in mind that the ESR spectrometer is not an analytical instrument of the spectrophotometer type (here a comparison of this kind has been attempted). A correct analysis of the ESR spectra would provide unique information in regard to the places and degrees of localization of the unpaired electrons. The main parameters are the width, form and position of the lines. As an example, let us examine the more detailed work of Alexander and Bacq who have read papers at this symposium. It is impossible to draw any conclusion as to the absence of localization of a free valency in a sulphur atom on the basis of a difference of the external form of the ESR line—of the irradiated serum albumin and of the cysteine.

The fundamental proof of the localization of an unpaired electron in sulphur lies not in the external shape of the line but in its position, in the magnitude of the  $g$ -factor.

It is well known from experiment and ESR theory that in the localization of an unpaired electron in the light atoms such as oxygen, nitrogen, carbon and hydrogen, the  $g$ -factor cannot be distinguished from the  $g$ -factor of a free electron by more than several units in the third place. At the same time, in localization in sulphur, the displacements of the  $g$ -factor exceed this value more than ten times. Unfortunately, the literature quoted does not provide the appropriate data. As far as an alteration in the resolution of the lines at different temperatures is concerned, various causes may be responsible for this.

It is, of course, possible that many injuries to biological macromolecules caused by ionizing radiation are not connected with the appearance of unpaired electrons. In this case, the ESR method can be of no assistance. The value of the method is determined to a considerable extent by the correctness of the formulation of the problem. We ourselves in our work have used irradiation only as a method of producing unpaired electrons in the structure and have utilized the valuable information which the ESR method has afforded.

It is doubtful if one can state today whether the excitation "migrates" along the structure and then leads to the disruption of a definite chemical bond or whether the unpaired electron already formed

migrates to a "weak" position. This question has not yet been solved in radiation chemistry, let alone radiobiology. The ESR method only gives the properties of the unpaired electrons already formed. Both may take place. One can, however, draw this conclusion from the experiments, that for this migration the conservation of the native structure—of the regular network of hydrogen bonds—is necessary. I think that the main possibilities for the ESR method in its application to radiobiological, and to biological, problems in general lie in the possibility of studying the weak, non-energy-controlling interactions which play such an important part in biological processes.

POWERS: It seems to me that it is absurd to suppose that ESR as an analytical method can show us why irradiation produces injuries in cells. But at the same time, one should not neglect that assistance which the ESR method may provide in determining what these injuries are.

It is absolutely clear that a penetrating radiation produces in a cell a whole series of effects. The ESR method, like spectrophotometry, is not suitable for the study of all the physical and chemical changes in cells.

It is extremely important to apply all the physical methods which are at our disposal. There may exist excited states of a radical and other kinds of effects about which we still know nothing.

It seems to me that in our work with the spores of bacteria we have correctly applied the ESR method. And further, we have indeed studied the correlation of the changes of the ESR spectrum with the corresponding specific injuries due to irradiation.

POLLARD: I will concern myself with two points. The first is the importance of the ionization processes in comparison with excitation; the second is some new data on the value of the S-S bonds in the irradiation of proteins.

The work of Hutchinson with bovine serum albumin has shown that no inactivation is observed below 11 eV, although this energy is much higher than is necessary for excitation.

Subsequently, the work of Setlow on u.v. irradiation in a vacuum showed that the quantum yield does not increase and does not approach unity provided that short waves of approximately 1,300 Å are not used. This radiation can ionize. Thus ionization is 100 times more effective than excitation.

In regard to the second point: the enzyme, ribonuclease, in the native state is not digested by trypsin. If it is subjected to irradiation, then



the trypsin acts. A study of the products of the digestion of the irradiated ribonuclease by paper chromatography, shows that they are the same as are obtained with the digestion by trypsin of the chemically restored ribonuclease. This is the work of Rey, Hutchinson and Horowitz.<sup>†</sup>

GRAY: The point is that the ESR measurements are carried out several minutes after the irradiation. They can be compared quite correctly with the results of the irradiation of spores about which Powers spoke. We know, however, that in normal cells with a large water content many reactions are carried out in a fraction of a second. In order to compare the results obtained in such material with the ESR data we must wait until we can carry out the measurements of the paramagnetic resonance within a very short period of time after the irradiation.

ALEXANDER: Blumenfeld states that our ESR data do not exclude the possibility that the electrons in proteins wander to the sulphur atom. He states that the dissimilarity between protein and cysteine patterns may well arise from secondary factors. I, unlike Blumenfeld, am not competent to deal with these fine points of ESR, but I would like to point out that our data do invalidate the argument used by Gordy and Shields to establish that the electrons wander to the sulphur in proteins. Their argument rested entirely on the fact that the ESR of irradiated protein and of irradiated cysteine were similar. We have now shown this similarity to be an artefact. Our chemical data indicate that there is no preferential attack on cysteine and it is now up to the people who claim that there is a localization on the sulphur to provide the evidence.

Pollard states that in his laboratory it has been found that the sulphur in proteins is attacked preferentially by radiation and he attributes the inactivation of enzymes to this preferential attack on the sulphur. We have published a detailed investigation of the effect of radiation on serum albumin in which we have shown that the cysteine residues are not exceptionally sensitive. Naturally, they are attacked together with all the other amino acid residues and the fact that a protein like ribonuclease which contains a lot of cysteine should have some of this altered, does not provide evidence for migration of energy to the cysteine. Our most extensive data is with serum albumin, but we have made similar observations which show that the cysteine is not exceptionally sensitive in  $\gamma$ -globulin, trypsin, lysozyme and keratin.

<sup>†</sup> *Nature, Lond.* (in press).

BACQ: II. Mitosis is more radiosensitive than DNA synthesis, but these two processes are not necessarily connected. Would anyone who does not agree with this care to comment?

HOLLAENDER: It seems to me that this question has not been correctly formulated. Only certain stages of mitosis are very sensitive. The later stages of mitosis are more resistant than DNA synthesis. Both these processes are unavoidably connected, because mitosis depends upon DNA synthesis. But it seems to me that this problem has been formulated in a topsy-turvy manner.

I would like to emphasize one point which Gray touched upon. One has to work very rapidly in order to study the first stages of the radiation effect. Within 60 sec mitosis can be so far advanced that we can no longer do anything.

SOŠKA: Karpfel in the Institute of Biophysics at Brno has found new criteria for determining the sensitivity of mitosis to irradiation. He has discovered in the cells of mouse bone-marrow that after irradiation the ratio of the number of metaphases to the number of prophase is altered. The normal ratio, which is 0.5, is raised after irradiation to 1 to 2. This variation proceeds even at very low doses, about 5 r, but only if the irradiation has been carried out between 15 and 19 hr prior to mitosis, i.e. the most decisive interval of time before the beginning of mitosis.

TOBIAS: I would also like to disagree with the formulation of the problem as put forward by the President.<sup>†</sup>

What is mitosis? Mitosis is the cellular activity by which the DNA of the mother cell transmits its information to the daughter cell. The time required for mitosis expresses the time necessary for the transmission of this information.

Thus we can examine the delay in mitosis caused by radiation either as a defect in the information, the source of which is the DNA, or as a defect in the channel capacity which is apparently a cytoplasmic process.

One of the interesting observations which has been known for the last 20 years consists in the fact that if mitosis is delayed for a certain prescribed period of time, then the cell in general cannot divide. This is seen from the work of Henshaw on sea urchin eggs, and has been quite recently shown in Bird's dissertation in our university on yeast cells after irradiation.

<sup>†</sup> From the Praesidium: the President said that he ventured such a formulation on purpose in order to provoke discussion.

BACQ: In many cells the synthesis of protein and nucleic acids is continued and at the same time no mitosis takes place. Such giant cells have been described by a great many authors, for example, Puck on cultures of Ehrlich's carcinoma. The presence of giant cells is revealed under the microscope. These observations confirm the formulation as I have already expressed it.

BARENDSSEN: I would like to draw attention to the fact that chromosome replication is necessary for the beginning of mitosis, but if only one of the chromosomes is not replicated mitosis can be inhibited. In contra-distinction to this, in order to inhibit the synthesis of DNA, a considerable amount of it must be damaged. I have shown that if only one  $\alpha$ -particle penetrates into a nucleus, then reduplication of the *chromosomes* is inhibited.

The inhibition of DNA synthesis in these conditions does not occur because only an insignificant part of the DNA is affected by one  $\alpha$ -particle.

May one regard these two processes as a single phenomenon? The mitosis delay process is much more sensitive.

BACQ: We must not allow any kind of confusion to arise during the discussion. The reduplication of chromosomes and the synthesis of DNA are two quite different matters. Moreover, Gray has said that it is necessary to pursue the distinction between the time delay of mitosis and the constant inhibition of mitosis. The giant cells about which I spoke are an example of a constant, long inhibition of mitosis.

ALEXANDER: Is it in fact true that the doubling of each chromosome is necessary in order that a cell may divide? In many cancerous cells chromosome abnormalities are observed with almost every division. But nevertheless these tumours grow very well. This has been demonstrated by the cytologist, P. C. Koller.

HOLLAENDER: I pointed out in my paper that a hypotonic medium may cause mitotic delay and even in general stop mitosis. This means that at the basis of the inhibition there may lie a very simple physical process. And I think that physical chemists and scientists interested in the permeability problem could take part in the solution of this problem. Such a simple process as mitosis delay in a hypotonic medium has not yet been investigated from the physico-chemical point of view.

ERRERA: The difficulties of studying the primary causes that delay mitosis are connected with the fact that most biochemical processes are inhibited much earlier than the time of discovery of mitotic inhibition,

It is difficult to recognize what is the biochemical process and at what time mitosis is initiated.

In the first place, it is necessary to look into the interconnections of the biosynthesis of protein, RNA and DNA. This is connected with the problem of RNA division between two daughter cells when the nucleolus dissolves (given the dissolution of the nucleolus). The formation of the spindle has also up to now not been sufficiently studied.

We do not as yet know what takes place in the formation of the spindle. Is this protein reorganized or synthesized?

And finally, it is necessary to study the connection between cytoplasmic processes and nuclear processes. In certain cases, the cytoplasm may exist and even divide in the absence of a nucleus. Thus the inhibition of division of the cytoplasm may depend upon the organelles of the cytoplasm.

BELGOVSKAYA: The synthesis of DNA and mitosis, and even such a simple means of cell division as amitosis, are not necessarily connected.

After the irradiation of *Actinomyces* the synthesis of the DNA is rapidly restored, but the division of the nuclei is not restored. Sometimes nuclei reach colossal dimensions and contain an enormous amount of DNA, but they do not divide.

The mechanism of mitosis is indissolubly connected with cytoplasmic structures, as Errera has said. But we have, however, every reason to suppose that the functions of these cytoplasmic structures are under the control of the cell nucleus. The following facts testify to this. The cleavage of fish eggs containing nuclei irradiated with a dose of 40 r proceeds normally up to the late blastula stage, while the cells contain the products which precede the activity of the nucleus. But when these products are exhausted, mitosis in the cells quickly ceases and the cells perish.

HOLMES: It is known that mitosis can be inhibited by means of a mechanism not connected with the inhibition of DNA synthesis. Small doses of X-rays stop cells going into mitosis even when the irradiation is carried out after the completion of DNA synthesis in those cells. We shall illustrate this situation by reference to the regenerating liver of rats. When proliferating tissue is irradiated with 450 r the advance into mitosis of all the cells is rapidly inhibited but I do not know the duration of this effect.

On the other hand, a 450 r dose given to the cells before the beginning of the DNA synthesis caused a delay of the beginning of the synthesis amounting to 12 hr, but did not alter the form of the DNA synthesis curve, nor did it alter the time relationship between the peak of the



synthesis and the beginning of mitosis. In fact, a further delay in mitosis was not observed. This means that the DNA synthesis and the mitosis were only postponed by 12 hr. But it does not mean that the DNA was not damaged, because in a large percentage of the cells embarking on division chromosome breakages were discovered (see the data of Koller).

BACQ: III. We now have to discuss the question of the influence of various factors on the repair of nuclear injuries.

TUMMERMAN: The question is completely incomprehensible. Wherein lies the difference between a natural metabolic process and an artificial process?

BACQ: I will be glad to explain this to you.

If an increase in the frequency of mutation depends upon the power of the dose, this in my opinion proves that when the radiation is of low intensity repair of the damaged genetic material occurs.

And once more I have put forward a somewhat provocative notion. Dr. Hollaender does not agree?

HOLLAENDER: I want to say that, in the phenomena discussed by us, repair of the genetic material does not occur. But is there an interference in the chain reaction leading to the appearance of mutations?

There is one point. Many potential injuries have been shown in experiments with i.r. rays, which by themselves do not have any marked effect, but in combination with X-irradiation augment the effect.

DUBININ: Some critical remarks on the problem of repair. Experimental data on the processes of genetic repair (after fractionated small doses of radiation) has been presented by Nonbak and Auerbach, and also by Luning on *Drosophila* and by Russel on mice. The new data in this case consist in that the dependence of the frequency of the mutations upon the intensity of the radiation has seemingly been shown in a group of mutations which are recognized to be gene variations, whereas this dependence was known only for chromosome recombinations.

If we consider the latter, then the restoration of the original structure of the ruptured chromosome can be carried out by two, in principal different, ways. Either it is a repair of the primary damage when the potential rupture is not realized, or it is a repair after the occurrence of chromosome breakage by combination of the fragments which have arisen.



It has been proved that the intensity of the radiation alters the possibility of the re-combination of the fragments. But for gene mutations we speak of the repair of the primary damage. While not objecting to the essence of the problem—the possibility of restoring gene mutations—it is nevertheless appropriate to ask if this phenomenon has been proved in experiments on the alteration of the radiation intensity.

In work with *Drosophila*, Luning and others used recessive lethal mutations. At the same time, Dubinin, Khvostova and Mansurova studied these mutations from the cytological point of view and showed for example that for a dose of 2 kr about 30 per cent of the lethals are connected with large-scale chromosome re-organizations. Only some of the mutations in Luning's experiments were capable of genetic repair. What is this part? Can it be that these are precisely of the category of chromosome arrangements? No analysis is available of this question.

Russel in his well-known experiments which have been described here by Hollaender dealt with a family of loci in mice, which gave recessive mutations with visible manifestations. They are recognized as gene mutations. But is this so in all cases? There is no reason to doubt that in this case the occurrence of many mutations is connected with chromosome rearrangements.

It has been shown in work with *Drosophila* that many mutations previously regarded as classic examples of gene mutations, (e.g. yellow, white, etc.) which arise after irradiation, in many cases turn out to be connected with chromosome rearrangements. Certain hereditary illnesses in man which have been regarded as gene mutations have also proved to be connected with chromosome rearrangements.

According to Russel's data, amongst the mutations studied, about half fall into mutations of one locus "S" which possesses both recessive and dominant manifestations. In the case where the mutations in this one locus are connected with chromosome rearrangements, the dependence upon the intensity of radiation, which does not concern gene mutations, can be connected with chromosome rearrangements.

Russel has not studied the cytological nature of the radiation mutations obtained by him in mice, and so the question of the repair of gene mutations in conditions of low radiation intensity remains open. This question is so important that it is urgently necessary to force a solution by using the category of true gene mutations. It must, however, be said that this is a very difficult experiment.

During recent months I have studied the influence of streptomycin on spontaneous sex-linked recessive mutations in *Drosophila* and on the spontaneous emergence of chromosome arrangements in the cells of

onion rootlets. In *Drosophila* under controlled conditions  $0.41 \pm 0.008$  per cent mutations are obtained. Under the action of small concentrations of streptomycin on ripe sperm,  $0.10 \pm 0.02$  per cent mutations occurred. In the onion rootlets in controlled conditions there were  $1.83 \pm 0.3$  per cent chromosome arrangements and under the action of streptomycin  $0.17 \pm 0.17$  per cent. Thus, in a natural mutation process the action of streptomycin is to repair. In this case there occurred, as it were, a "healing" of the hereditary substance which is accomplished at the molecular level.

In conclusion, I must say that even taking into account the repair phenomena the following basic principles hold even for the case of genetic effects:

1. The absence of a threshold radiation dose.
2. The existence of a direct dependence of the frequency of the mutations upon the dose, and,
3. The cumulative effect of small radiation doses.

All this leaves the stable position that the influence of any, including all, small radiation doses is for a human being genetically harmful.

HOLLAENDER: Russel has data that show that true gene mutations occur in the "S" locus. I would like to suggest that there is one further consideration. Russel has never claimed that he can repair gene mutations. But he has dealt with a process which leads to the appearance of mutations. The cytological work connected with these problems is now being carried out. In the next six months the opportunity will occur of reporting the results.

We can see from Dubinin's contribution that he is also working on this problem, but he is dealing with another type of organism and the further development of this work will be of very great importance.

ERRERA: I would like to say a few words concerning cytoplasmic repair.

Brachet has shown that cytoplasm in the absence of a nucleus is capable of being restored, but only for a very limited period of time. Consequently, damage to the nucleus is important also for the restoration of cytoplasmic lesions in the cytoplasm. This is very well demonstrated by the ageing of irradiated cells.

BACQ: IV. Chromosome breaks are not only the result of direct radiation effects. They are the result also of secondary effects and can be modified by the metabolic activity of the cell.

SHAPIRO: I do not agree that chromosome breakages are a secondary effect. The chromosome breakages, and also point mutations, are a primary and local process.

At the present time the presence is presumed of two forms of primary variation of the chromosomes, real and potential. The potential variations in certain circumstances can be reversible or transformed into real mutations.

This process of reversal of potential lesions is a secondary process that depends upon the metabolism of the cells. Thus, it is necessary to distinguish the injuries to the chromosomes, which are primary processes, and their realization, a process both secondary and dependent upon the metabolism of the cell. The inadequate definition of the restrictive nature of these two processes is reflected as it seems to us in those sentences which Bacq formulated for discussion: III the nuclear variations were regarded as primary, in IV the nuclear variations were regarded as secondary.

The existence of potential variations and the dependence of their fate upon the cellular metabolism complicates the study of the primary process. But this does not mean that the process of damaging the chromosomes is not primary.

BACQ: When someone studies under a microscope the rearrangement of chromosomes, he is studying cells which have been irradiated up to the point of entry into the prophase. You cannot see the re-arrangement of chromosomes before the cell enters into metaphase or anaphase. A cytologist cannot see what transpires between the instant of irradiation and the metaphase stage. He therefore erects a hypothesis in regard to phenomena which have taken place prior to the stages actually observed by him.

However, the metabolic processes are continued throughout that period of time when, properly speaking, the cytologist cannot see anything.

GRAY: The remarks which have just been made by Bacq indicate that metabolism has possibly already completed its work. This cannot be excluded.

In some cases the amount of observable variation in the chromosomes depends upon the metabolic processes which occur between irradiation and observation. But not all stages of chromosome damage, and in particular not all types of damage, are under the influence of metabolism. This can be proved. Therefore, it still remains an open question, whether certain chemical reactions are capable of bringing about directly the destruction of the chromosomes.

BACQ: V. Is there a series of primary chemical injuries which is correctly regarded as damage to the structures (membranes, intracellular structures) and is the destruction of the intracellular auto-regulation as a consequence of the action of an ionizing radiation?

SHABADASH: By investigating, over the course of many years, the consequences of radiation effects at the cellular level and on the organism as a whole, we have succeeded—by means of histochemical tests—in demonstrating the following. Cytological variations occur in all the cells (both sensitive and resistant) and in addition the non-identical finite result of the reactions depends not only upon the constitutional properties of the type of cells, but also upon the intracellular regulating influences if the matter concerns the systems of the organism. A sensitive indicator of the cellular variations is the destruction of the physico-chemical properties of the nucleoproteins of the mitochondria, which reflect the breakdown of the metabolic nuclear-cytoplasmic interaction. The phenomenon of multiplication and amplification of the primary ionization effect, which arises even at the cellular level undergoes a modification, and is reproduced in a complex organism through its nervous system. It is known that sensitivity to radiation damage increases in the evolutionary series; from our point of view this phenomenon is governed by the development and perfection of the nervous system in all the variety of its effects.

Our experiments with irradiation were undertaken on male rats, using a dose of 50 to 100 r and a dose-rate of 150 to 192 r/min. The criterion of the reaction of the nerve cells was the displacement of the iso-electric point (IEP) of the ribonucleoproteins (RNP) which testifies to the destruction of the normal interrelationships of the enzymes and substrates and which is accompanied by a considerable decrease of the normal thresholds of irritability. The animals were fixed totally by an injection through the vascular system after 1.5, 10, 20, 30, 45 and 60 min; and then, after 1, 2, 3, 4, 6 and 24 hr, and every 24hr (for 10 days). The greatest alterations were discovered in the ganglion cells of the spinal cord, the displacement of the IEP in the mitochondria of which, even in the first minute after irradiation, reaches (according to the logarithmic scale for pH) 0.9 to 1.0 units, i.e. is of the order of 25 times the absolute variation; the displacement increases after 5 and 10 min (to 1.2 to 1.4 units) and, beginning from 20 right up to 60 min, is somewhat reduced, but it is very far from returning to the original value; after 24 hr (and then for all the first week) there is observed a wave-shaped shift of the oscillations of the IEP values. Analogous although quantitatively somewhat different,



displacements of the IEP and pH of the mitochondria take place in the neurons of the cortex and—the least—in the motor cells of the spinal cord. It is important to emphasize that the destruction of the physico-chemical characteristics with pH of the mitochondria is observed when the entire morphological structure is maintained, being evidently an index, and a reflection, of the functional pathology of the cells.

We ascertain: (a) the appearance of extensive cytochemical variations in the afferent nerve cells of mammals in the irradiation process which may be recorded in the first minute after the action; (b) these destructions, which are amplified or weak or damped in a wave-shaped manner, are maintained in the first hours and time periods, i.e. during a latent period; (c) they are accompanied by analogous increasing cytochemical and functional injuries of the higher centres of the vegetative system (the intermediate brain, the reticular substance, etc.) and, partly, of the cortex of the hemispheres (cerebral hemispheres) which must be considered responsible for the generally known manifestations of radiation sickness, for example the destruction of mitosis and regeneration, the derangement of blood circulation and the blood system, etc.; (d) in the course of the disease, there arise paraneurotic and thereafter degenerative conditions of the neurons.

We, sharing the opinion of Livanov, Bryukov, Livshits, Grigor'yev, Arbuzov and others, believe that in higher vertebrates the radiation damage must be estimated as nerve damage, the individual links of which are accessible for radiation and aetiological therapy.

It is necessary to distinguish and separate the leading elements of radiobiological processes which are sharply dissimilar at the cellular level and in systems of the whole organism: the elementary mechanisms at the cellular level prove to be, for the most part, similar (in spite of the properties of diverse types of cells and the final outcome of the effects caused by them); but from the moment of the emergence of a nervous system, and as its functions develop and become more complex, the leading element of the radiation damage consists in the destruction of the nerve regulations, which comprise the direct reflex and metabolic reactions. In other words, in the process of evolutionary complexity a shift occurs in the "control" systems, which, in any case for vertebrates, brings to the fore the "supercellular" correlation of the organism as a whole, i.e. its nervous system in the Pavlovian sense of its function as an equilibrator with the external medium; given adequate compensatory opportunities to the nervous system, even the initial radiation variations do not occur without a trace.



In recent years many competent radiobiologists have begun to recognize the important part played by the biological properties of living organisms in the features of post-radiation processes. Thus, Rayevsky stresses "the necessity of recognizing the physiological processes in the cell in order to explain the biological action of the radiations". Baeq recognizes the occurrence of reciprocal reactions of the nerve cells and fibres under radiation, although he supposes their subsequent levelling (which in reality does not occur). Many rapid reflex manifestations in invertebrates have been shown. Summing up our data, (part of which, concerning the rapid occurrence of histochemical and functional destruction in the central nervous system, has been quoted above) and the results of other authors, we suggest that evolutionary biology, alongside an understanding of the radiophysical and radiochemical nature of primary processes, obliges us to recognize a change in the nervous system as the principal mechanism for the beginning and development of radiation sickness of animals and of man.

ALADJALOVA: I will concern myself with the question of the role in the radiation effect of intracellular surfaces, and intracellular structures, and of the conservation of the damage by the investigation of ion transfer in the insulated muscle of a frog.

The data were obtained from measurements of the dielectric losses of the tissue at audio and radio frequencies which served as the ion transfer index. The irradiation was carried out in Ringer solution by X-rays in doses from 5 to 100 kr; the dose-rate was 700 r per min.

The direction of ion movements in the muscular tissue after irradiation by X-rays (in the "closed" period with respect to the physiological indices) is different from the direction in the stage of the manifestation of the biological effect. The variation of the dielectric losses in the muscle even begins (see Fig. 1) during the irradiation and, from the start, constitutes a fall. This can be explained by a reduction in the number of ions in the tissue, which participate in the electro-conductivity. In the phase of the fall in transfer the excitability of the muscle is maintained, moreover it is sometimes even increased—this is the "concealed" period.

However, after a certain dose which we shall call the "turning point" (we speak here of doses of the order of 40 to 50 kr, for the skeletal muscle of a frog) an increase in the dielectric losses in the muscle occurs: the development of ionic processes of opposite direction (polarity) is initiated. In this period the overall electric charge in the system is increased due to the liberation of a large number of ions from the surfaces of the membranes, evidently due to the beginning

of the destruction of the latter. The excitability of the muscle progressively falls—this is a “clear” period.

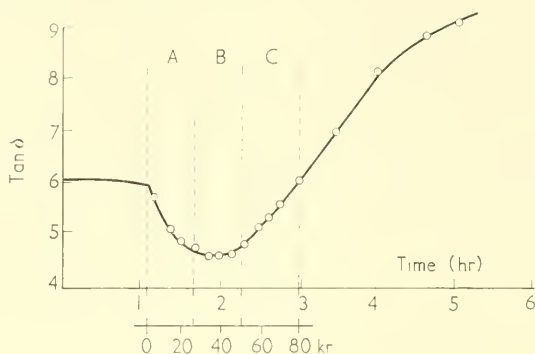


Fig. 1.—The variation of the dielectric losses in the tissues of mice in the process of irradiation with X-rays.

The turning-point dose, after which the direction of the physico-chemical processes varies, is higher if the irradiated structure possesses more highly differentiated biological properties which probably correspond to a greater degree of macromolecular order. For example, the following relationship is worth noticing: the stronger the electric polarization at the boundary surfaces, the higher the “turning-point” dose. For smooth muscle, the electric polarization at the interphases is lower than for skeletal muscles, and correspondingly, for smooth muscle the turning-point in the loss curve occurs at lower doses, (12 to 30 kr). The impression is created that the degree of ion concentration at the interphases is indirectly connected with the factors which influence the radio-resistance of the structure.

On the basis of an analysis of the loss curves at various frequencies, one may think that the mechanism of ion displacement in the structure of the skeletal muscle in the “concealed” period lies in the amplification of the ion concentration at the interphases. In connection with this it is interesting to note that Alexander and Charlesby have observed a phase of increase of the polymerization of synthetic polymers after  $\gamma$ -irradiation within a definite range of doses.

We have also obtained a proof of the dependence of the radiation effect upon the order of the structural construction of a muscle in experiments with the irradiation of the denervated skeletal muscle of a frog. A few days after cutting the nerve, the distribution of the electrical charge in the muscle is altered on the side of a weakening of the ion concentration at the polarized boundaries, and the reaction of the

muscle to chemical factors is also altered in the direction of a decreased sensitivity; simultaneously, the turning-point dose is also decreased—it is moved from 50 to 35 kr.

To transfer the ion displacements of one polarity into another will require either a sufficient irradiation dose or the inclusion of additional factors, in particular, an effect by i.r. rays following the X-rays, which facilitates the effect of X-irradiation, and which is not shown after the action of X-irradiation alone.

In these experiments the thermal effect of the i.r. illumination is as far as possible eradicated by special filters and by the replacement of the physiological solution. The i.r. radiation being applied as a separate factor does not cause any variation of the electrical properties of the muscle. The action of the i.r. radiation 2 hr after the X-ray irradiation halted the development of the displacements in the electrical properties of the muscle, which are observed after X-ray irradiation, i.e. tended to conserve the macromolecular complexes. These data are, in principle, in agreement with those of Hollaender, although they were obtained for different objects. According to our data, the i.r. irradiation provokes for the most part that mechanism which determines the low frequency electrical conductivity, i.e. affects the conditions for the ion concentration at the polarized interphases. The degree of conservation is connected with the original construction of the surfaces of the membrane and is increased in the more complex structure of the skeletal muscle in comparison with smooth muscle.

HUG: I would like to make several comments on the connection of the problem of permeability with the action of radiation.

Unfortunately, up to the present time there is only a small number of direct proofs of the variation of the permeability in radiation conditions.

Only after irradiation with very high doses is a loss of potassium ions observed. This, however, can be explained by the fact that an unsuitable method and objects were employed. Permeability should be measured during the irradiation period and the kinetics of this process must be studied.

From the biophysical point of view one must expect here a strong dependence upon the power of the dose and upon the linear energy transfer. It is necessary to conduct new experiments and at the same time to examine the old work from a new critical standpoint.

Permeability is a far too general term. The difference in permeability of the membranes for different substances is extremely large. The permeation of inorganic substances must be distinguished from

that of organic macromolecules with diverse properties. One can obtain variations in the permeability by diverse means: by an alteration in the solution, an alteration in the Donnan equilibrium, and on the other hand as a result of a variation in the biochemical properties of the membranes themselves.

The question of the permeability of a cell cannot stand in isolation, because the cellular membrane with its highly organized structure has a direct relation to this problem. The external cellular membrane consists for the most part of lipoproteins and if irradiation acts on this cellular membrane, then identical variations under the influence of radiation must also arise in many other intracellular structures identical in respect of their ultramicroscopic structure.

BACQ: I agree with Hug. I have not used the term "permeability". I know many cases where negative results in regard to the variations of permeability in irradiation conditions have been discovered. For example, the gills of the freshwater crab absorb potassium and sodium from the surrounding medium. My colleague, Ghosh, of Louvain University, could not find any variations of the permeability after irradiation, but the conditions of the experiment were such that one would be able to carry out observations during irradiation.

MEISSEL: I would like to draw your attention to two facts. The first is this: in the structure of mitochondria there are portions that are more sensitive and portions that are less sensitive to radiation effects. Those enzymes which are connected with mitochondrial membranes and with the cristae of the mitochondria are as a rule extremely radio-resistant. The enzyme systems which are located inside the mitochondria are much more radiosensitive and the most radiosensitive systems are those which are connected with the structural integrity of the mitochondria as such. Consequently, the radiation damage to the membrane and the membraneless mechanisms of the mitochondria has considerable significance for an understanding of the initial radiation effects.

A second comment: Shabadash, in his contribution, rightly directed attention to the value of the destruction of the mutual relationships between mitochondria and the nucleus of the cell which occurs as a result of irradiation.

I would like to direct attention to the destruction of the interconnections between the mitochondria and the endoplasmic reticulum which unquestionably arises under irradiation conditions and which is expressed by the fact that, even for comparatively small irradiation



doses, the regulating action of the mitochondria in the synthesis of fats and sterols is noticeably impaired, as a result of which a sharp increase in the synthesis of fats and sterols occurs.

I would like to emphasize that the radiosensitivity of this system (the interactions between the mitochondria and the endoplasmic reticulum) is almost the same as the sensitivity of oxidative phosphorylation and the metabolism of DNA. Thus, for example, in yeast a marked increase in the biosynthesis of ergosterol takes place after irradiation of the cells with a dose of 5 kr; at the same dose a reduction in the DNA metabolism is noted. All the remaining metabolic processes are much more radio-resistant.

More remarkable is the fact that the formation of sterols in irradiated cells, which continue to metabolize, increases. This is an extremely common reaction to irradiation, observable both in micro-organisms and in the cells of higher organisms.

MOUTON: I think that, in examining the damage caused by irradiation, we have somewhat neglected the fact that there are natural conditions, unrelated to radiation damage to the cell, where processes of metabolic damage may be produced which are also observed in radiation damage.

For example, the post mortem autolysis of cells which has been investigated in my laboratory is accompanied by a reduction of pH which evidently leads to the rapid destruction of the nuclear membrane and then to the activation of proteolytic enzymes.

With this in mind we have at the present time undertaken investigations which have as their purpose to compare autolytic and radiolytic lesions in mice, which are nurtured in aseptic conditions.

We would welcome the study of this question for other objects belonging to other biological systems (a study that could confirm or reject our hypothesis), that the damage to the structure by radiation can arise as a result of a rapid alteration of a metabolic process, which is similar to the normal glycolytic process observable in the natural death and physiological autolysis of the cell.

KUZIN: It seems to me advantageous at the end of our discussion to attempt to compare the diverse points of view expressed here and to present, as it were, schematically the most probable course of events arising in the irradiated cell. In 1958 I gave such a scheme and I will permit myself today to draw your attention to this, introducing into this scheme greater refinements on the basis of later investigations, because, as it seems to me, it very closely reflects our conceptions.



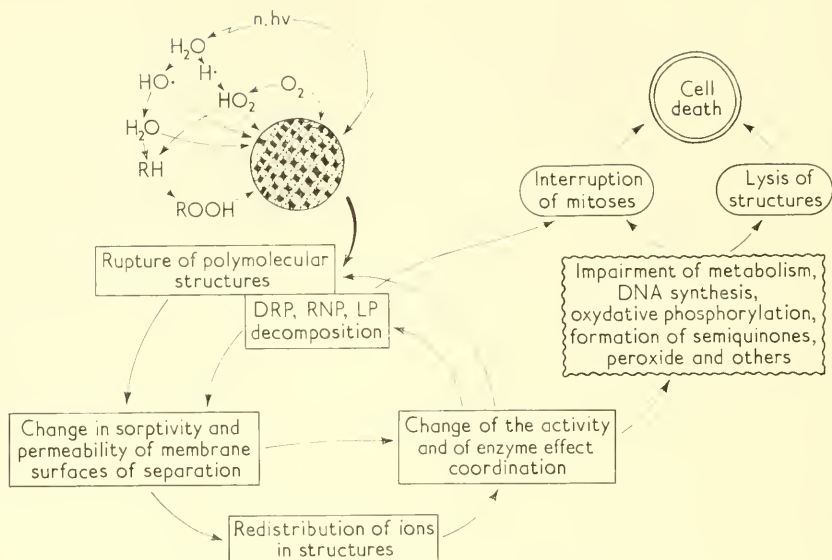


Fig. 2.—Scheme of initial processes in the irradiated cell.

I would like to emphasize a few isolated points only.

In the first place, of substantial significance for the fate of the cell is the radiation action not on the individual molecules of the substance, but on those complex, ordered, macromolecular structures which constitute the basis of the sub-cellular organelles of the cell. The migration of the absorbed energy through these structures, the occurrence of excited states of long duration (Powers, Eidus), the possibility of the occurrence in them of rapidly flowing short-term chain reactions (Tarusov), the deformation of considerable volumes of these structures, even as a result of the occurrence of single errors between the polymers which form them (Passynsky), all this forms the basis for an initial physico-chemical amplification of the radiation effect.

As a result of these initial processes, many physico-chemical properties of the elementary structures are altered in the first place, e.g. the permeability and the sorbability of the membranes. The importance of these changes was stressed in the papers read by Alexander and Baq, by Passynsky and in our publications in the years 1957–1958. A redistribution of ions (potassium, sodium, etc.) occurs. Many enzymes are activated. Even the slight activation of such enzymes as DNase, RNase and lipoproteinase results in more marked changes in the high molecular weight polymers of nucleoproteins and lipoproteins, which comprise the elementary structures of the cell and lead already to a

biochemical amplification of the radiation effect. In view of these secondary but very rapidly advancing changes, the physico-chemical properties of elementary structures are altered much more significantly as emerges in the variation of the iso-electric point of the nucleoproteins (Shabadash) and in the correlation of the free and bound lipids (Dœmin), and leads to a more significant change in the co-ordinated work of the enzymes, to an amplification of the activity of some and the weakening of others. The amplification of the enzymatic oxidation reactions, especially in the presence of oxygen, leads to an anomalous concentration of some active compounds (e.g. peroxides of unsaturated acids—Chevallier and Bacq—of active quinones, of semiquinones which easily give active free radicals—Kuzin) which complete destruction of the sub-cellular structures, inhibit and distort mitoses (Kuzin), convert the initial concealed injuries, for example in chromosomes, to manifest breaks (Hollaender, Shapiro). When the intensity of the processes described is adequate destruction of the metabolism of the cell proves to be so considerable that it will lead to lysis, to the death of the cell. This increment in the destruction of the individual exchange reactions, will in such a case be of an avalanche nature, and will be formally described by those equations which are applicable to chain reactions in purely chemical systems. All the conditions which delay the unfolding of this metabolic chain will delay the transition of the concealed damage to open damage, amplify the probability of the return of the initially destroyed structures to the initial state, and by this very fact assist the conservation of the cell. This probably accounts for the mechanism of action of many protective substances. (Bacq and Alexander, Shapiro, Hollaender, Grayevsky).

**ZEITLIN:** In DNA solutions under the influence of various factors (the binding of proteins or amines to the DNA etc.) there is observed in many cases a reversible two-fold drop in the characteristic viscosity without a variation in the molecular weight. Therefore, this variation in viscosity may be treated as a consequence of a change in the configuration of the DNA molecule, possibly connected with a reduction in the degree of symmetry which somewhat tentatively we shall call "compression".

We have studied jointly with Spitkovsky and Tongur the radiosensitivity, for moderate radiation doses, of native DNA and also of DNA in which compression has occurred. We have studied the behaviour of DNA in dilute solutions in which intermolecular interactions can be excluded.

DNA in a compressed condition proves to be much more radio-resistant than ordinary DNA. For doses of 5 kr, the fall in the viscosity

of the DNA solution amounts to about 50 per cent, whereas for DNP, DNA-CH<sub>3</sub>NH<sub>2</sub> and DNA at pH 2.8, a drop in the viscosity is scarcely observed. DNA with a molecular weight of 10<sup>6</sup> or lower is also radio-resistant. The latter were obtained by splitting the high polymer DNA by Doty's method. A spectrophotometric investigation of this DNA has shown the absence of differences in the H-bond system in comparison with the initial DNA with a molecular weight of 6·10<sup>6</sup>. At the same time many reactions, in particular the application of pH of up to 2.8, do not give rise to any drop in the viscosity of solutions of low molecular weight DNA. Thus, it turned out that in all the cases which were investigated, DNA suitable for "compression" proves to be more radiosensitive than DNA for which the "compression" occurred before irradiation due to preliminary action. It seems to us that we may draw two conclusions from this: (a) the alteration in the radiosensitivity of DNP in comparison with DNA is connected with the transition of the DNA under the action of the protein into a configurationally more stable state, and (b) comparatively small doses of radiation produce an alteration in the configuration of the DNA without a marked degradation. The essence of these changes evidently reduces to a natural (peculiar, intrinsic) non-specific "compression" of the DNA macromolecule.

As with the complex formation of DNA with protein there will arise not only a configuration change of DNA but also of the protein entering into the complex, the molecule of the protein being somewhat spread out on the surface of the DNA macromolecule. It became of interest to investigate the influence of the DNA on the radiosensitivity of the protein entering into the complex. This problem was studied with the use of artificial complexes of DNA- $\alpha$ -chymotrypsin and in addition it was shown that for doses of 10 to 20 kr for the  $\alpha$ -chymotrypsin in the complex with DNA there was a marked inactivation whereas for free  $\alpha$ -chymotrypsin no fall in the enzyme's activity is observed. The facts quoted demonstrate that complex formation in some cases reduces and in others increases the radiosensitivity of individual biochemical components.

KRITSKY: Tarusov and Mouton in their contributions mention the increase in the autolytic destruction of proteins after X-ray irradiation. An investigation which we carried out on the caterpillars of the bee moth after total X-irradiation by dose of 2 kr shows that autolysis is increased approximately twofold. The ATP content is reduced by 36 per cent and the total content of the nucleotides by 33 per cent. Thus the autolysis rate is altered to a greater extent than the concentration of free nucleotides.

In experiments on local irradiation of the bones of a rabbit by a dose of 2 kr the variation in the autolysis of the nucleic acids and also of the content of free nucleotides took place only in the irradiated extremities.

The autolytic fission of the nucleic acids in the irradiated bone-marrow may be seen from Fig. 3 to be reduced two or three times in 7 hr. The inhibition of autolysis of the nucleic acids within a long period of time is irreversible, whereas the content of the free nucleotides during this time approaches the normal.

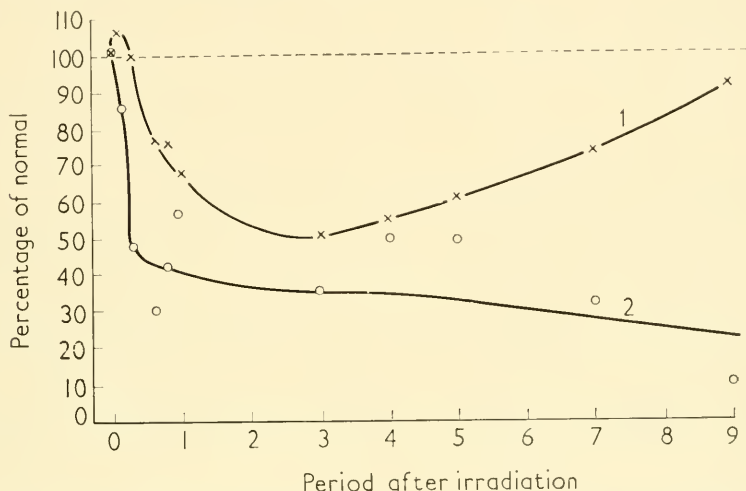


Fig. 3.—The variation of the rate of autolysis of nucleic acids and the content of the acid-soluble purine and pyrimidine compounds in the bone-marrow of a rabbit after local X-irradiation with a dose of 2,000 r.

1. The content of acid-soluble purine and pyrimidine compounds.
2. The rate of autolysis of nucleic acids in homogenates of irradiated bone-marrow.

It is evident that, within a very short time after X-irradiation the nucleic acids of the tissues are strongly broken down by nucleases. The result of this is that a smaller amount of nucleic acid will remain in the irradiated tissue. These nucleic acids, as Oparin has shown, will inhibit proteolytic enzymes. It follows that the cause of the increased autolysis of the proteins after irradiation is the reduction in the nucleic acids in the tissue

ASTAUROV: Experiments with irradiation of the nuclear and non-nuclear parts of a cell with accurately-localized application by means of micro-beams have shown that irradiation harms both the nucleus and the cytoplasm.

Although nuclear damage becomes dangerous for doses of between

100 and 1,000 times less than those which cause equally significant damage to the cytoplasm, the latter damage without doubt exists and plays an important negative role.

Amongst the multifarious initial injuries to the cell, it is quite possible that the destruction of the boundary cellular membranes plays a part. Alexander and Passynsky attribute great significance to this, although it seems to me that there is still much that is hypothetical and speculative.

The confused interaction of the initial reactions of the cell to radiation leads to an extremely complex picture.

We must attempt to separate, amongst the large number of initial variations, those which give rise to important secondary biological consequences and thus give to the radiation sickness of the cell, or of its multicellular organism, its specific properties, and, in particular, the difficult reversibility which is so characteristic of it.

The initial radiation injuries to the cell must be vitally important under at least two conditions: in the first place if they damage unique microstructures which occupy a key position in biosynthesis, the loss of which cannot be compensated by other apparently identical structures; in the second place, if the change which has occurred is irreversible and is reproducibly transmitted to daughter cells in the process of ontogenesis, or from one generation to another, continuing to give rise thereby to one and the same malfunction in this specific synthesis.

It is not difficult to see that these conditions are completely satisfied by that cellular apparatus which, by determining the processes of morphogenesis, secures the hereditary stability of the biological system; I have in mind the cellular nucleus, or more accurately, its genetic elements.

On the contrary, the cytoplasm of the cell is characterized by a multiple repetitiveness of its biochemical components, of identical macromolecules and microstructures; by lability and by intensive metabolic processes. In view of this it possesses the capacity for automatic regulation by adaptive and reparative reactions in response to damage inflicted from outside.

We must therefore expect a much larger reversibility of radiation injuries to the cytoplasm and their relative biological significance to be less than is in general justified by the facts.

Given such a functional approach the primary effect of the radiation or the chain of initial changes (if this is a chain) must acquire a particular biological significance, starting with the element which already affects the genetic structures of the cell.



There must be added to this the particular sensitivity of high-polymer DNA, and its synthesis, to ionizing radiations.

Therefore the key point in radiation injuries to the cell and in the search for the means of protecting it from such injuries, in my opinion, is the study of the biophysics and biochemistry of the processes which lead to the occurrence of genetic injuries to the chromosomes and the quest for means of liquidating these changes.

This latter problem appeared until quite recently to be hopeless and without any method of approach. Now, as we have heard, in particular in the contributions of Hollaender and of Shapiro, possibilities have emerged whereby these genetic processes may be affected at least at the very initial stages of their development.

ALEXANDER: The hypothesis which Bacq and I have advanced that an important type of radiation damage that leads to cell death consists of the disorganization of intracellular structures does not mean that we are only concerned with the cytoplasm. The electron microscope has revealed the existence of an extensive cyto-skeleton in the cytoplasm, though the prediction that such a structure must exist was made more than 30 years ago by R. A. Peters. He argued that the presence of enzymes and their substrates required that they be separated by fine lipid films. We now know that in the nucleus, there are also enzymes which must be kept away from other nuclear material which they can attack. Fine structures of the type seen in the cytoplasm must therefore also exist in the nucleus. The fact that these have not been revealed by the electron microscope can, I believe, be attributed to technical factors. Our theory that the mechanism of action for radiation in relation to cell death is therefore not confined to the breakdown of structures in the cytoplasm, but includes the breakdown of structures in the nucleus.

PASSYNSKY: Any general theory of radiobiological action must at the present time postulate the destruction of the cell through intermediate biochemical processes from the primary destruction of several molecules or of a certain volume of molecular dimensions. Damage to the chromosomes as the unique structures of the cell satisfies this requirement. The role of the damaged chromosomes in the genetic consequences of the radiation remains completely inviolate, but many papers at the present symposium and, indeed the last discussion, show that the concept of the primary nature of this damage is evidently in need of some modification. Another view has been expressed namely that at the instant of irradiation, some intracellular membranes are damaged.

This can be, in the first place, the nuclear membrane itself—then the data submitted by Barendsen are comprehensible, namely that injury to it in any place by one particle leads to loss of electrolytes and nucleotins and to the destruction of the biosynthesis of DNA, in consequence of which the damage to the chromosomes acquires a manifest character. Then the data of Barendsen, of Errera and of Hollaender are immediately explicable. There can be, in the second place, damage to the cytoplasmic membranes which facilitates the interaction of enzyme and substrate—and we shall then obtain the activation of a series of enzymes as is indicated in Bacq and Alexander's first paper, and in our paper. This may also explain the interesting data of Hug and the data of Kuzin which are in complete agreement with that obtained in our laboratory for the interaction of polyphenoloxidase with tannin in tea-leaves and of lipoperoxidase with unsaturated fatty acids. The primary function in this case is not the production of peroxides or semi-quinones but the destruction of some intracellular structures which facilitates the contact of enzyme and substrate. However, we have not studied alterations in mitosis, but I would like once more to emphasize the great significance and interest of Kuzin's data.

The damage to the enzymes is not evidently by itself a primary factor in radiobiological action. It may be assumed that the damage to the mitochondria is also not a primary phenomenon. There are thousands of them in the cell, and there is no foundation for the supposition that any one of these structures is unique. It follows that damaging them does not satisfy the primary criteria.

It is evident that in the cell there are only two kinds of uniqueness—the uniqueness of the chromosomes and the uniqueness of the flow of biochemical transformation, which is regulated by the nuclear, and by certain cytoplasmic, membranes. All the basic problems of radiobiology as shown in particular in the article which we published† can be explained by damage to these. The concept of damage to the membranes is connected in radiobiology with the general theory of open systems (but it does not form the basis of the self-adjustment and homeostasis of living organisms, about which Frank made some comment). There is no need to juxtapose chromosome damage and membrane damage. Both the studies on the structure of biopolymers and also the theory of open systems are internally connected and form the two physico-chemical bases of radiobiology.

TUMERMAN: Unfortunately, our discussion has diverged from consideration of the problem of the single nature or the plurality of primary

† *Biophysics* (Russ.), 1957.

processes of radiation damage. It seems to me that in order to reply to this question, it is necessary, with maximum clarity, to emphasize the distinction between the two types of radiation damage. To the first type we must refer various injuries which are restricted to the cell which has received the radiation, has had its structure, metabolism and function destroyed and, in the extreme case, has been brought to death: these injuries are not transmitted to its descendants. To the other type belong such injuries as do not lead to the death of the cell, but are reduplicated through many cellular generations. We are dealing here with a change in genetic information.

I cannot understand why the attention of radiobiologists in particular, and at this symposium, is to such a great extent concentrated on injuries of the first type, and why so little attention is devoted to injuries of the second type. In particular, in the general integration of radiation damage processes presented here by Kuzin, there is not even a place for changes in the code of the genetic information. This unequal distribution of attention between injuries of the first and second types is the less understandable in that injuries of the second type are not only incomparably more interesting from the general biological and evolutionary point of view, but also have much more practical value than injuries of the first type.

Turning now to the problem posed by Baeq, I would like to say that one can destroy or damage a cell by many methods, and therefore various chemical reactions or series of reactions may evidently be responsible for these injuries at the chemical level. As far as the variations which are reproducible in the descendants of the irradiated cell are concerned, then it is possible that there exists here some general mechanism for the variation of the genetic code. It is very probable that in all cases of the occurrence of mutations, under the action of diverse factors (ionizing radiation, chemical mutagens, etc.), the primary mechanism for the variation of the code is one and the same. It seems to me, partly intuitively—I have no proof of this—that we are speaking here not of general chemical reactions but of a certain common, elementary, physical action the mechanism of which is still not yet completely clear; as unclear, by the way, as the mechanism of coding and decoding of the genetic information itself.

It would be of great interest to me to know what biologists think about this question.

TRINCHER: Erythrocytes, suspended in a physiological solution which contained blood serum, proved to be the more radiosensitive the lower the concentration of the serum proteins. This phenomenon—a protective

action of the proteins in the external cellular medium—explains the high radioresistance of erythrocytes in the organism. It was of interest to investigate the effect of non-protein protective substances on the surface layer of the erythrocytes in the absence and in the presence of serum proteins.

Together with Kuzin we studied the action of the following substances: (1) cysteamine and  $\beta$ -aminoethylisothionuronium bromide (AET); (2) ascorbic acid and adrenaline; (3) morphine and nembutal; (4) glucose and blood serum (Fig. 4). The action of these substances is expressed by the long delay before the beginning of haemolysis in the isotonic alkaline buffer (pH 9.97) in the presence of a protective substance in comparison with the time of haemolysis in the control experiment.

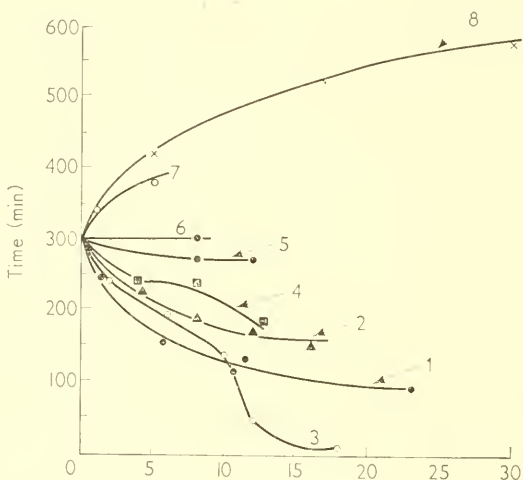


Fig. 4.—The dependence of the time of initiation of haemolysis upon the concentration of the substance.

1, cysteamine; 2, AET; 3, ascorbic acid; 4, adrenaline; 5, nembutal; 6, morphine; 7, glucose; 8, serum proteins.

Curves 1–6, at 10 mg/ml;

Curves 7 and 8, at 1 mg/ml.

As may be seen from the shape of the curves, cysteamine, AET, ascorbic acid and adrenaline have a strong effect on the surface layer of the erythrocytes. With the action of these substances on a cell in a protein-less solution there occurs an injury to the surface layer of the erythrocyte which is revealed in the sharp reduction of the time of initiation of haemolysis. Neurotropic substances—nembutal and morphine—prove to have a weak effect on the stability of erythrocytes.



Glucose and serum proteins prove to have a strong protective action on erythrocytes. Glucose and serum proteins in increasing the stability of the surface layer of erythrocytes, also possess a clearly defined radiation protection.

It was of interest to investigate by this same method the joint action of cysteamine (AET, ascorbic acid, and adrenaline) and of serum proteins on the surface layer of an erythrocyte. Experiments showed that the addition of serum proteins reduces or completely removes (given a sufficiently great concentration) the harmful action of the cysteamine, (or AET, or ascorbic acid, or adrenaline) on the surface layer of the erythrocytes.

The serum proteins protect the surface layer of the erythrocytes both from the damaging action of the radiation and also from the damaging action of these substances.

MANOILOV: I would like to touch on the question of the primary changes that take place in the cells of a living organism under the effect of radiation. In the beginning it seemed that the most radiosensitive substances in the cells of living organisms were nucleic acids and nucleoproteins.

In Alexander's interesting paper, and also in many other communications at this symposium, it has been shown that changes in the nucleoproteins cannot be responsible for the development of radiation injury in the cells. In his paper Alexander put forward the supposition of the special sensitivity of the phospholipids which enter into the composition of cellular membranes. Unfortunately, the experimental proofs on behalf of this supposition were not given in the paper.

In the course of the last ten years we have developed the point of view that the most radiosensitive substances in cells are the chromoproteins, or speaking more accurately—the variable valency metal-containing enzyme systems. Amongst them we include the cytochromes and other iron-containing enzymes.

It is known that these substances participate in processes connected with the liberation of energy. It has already been noted for a long time that after irradiation of the living organism, a sharp reduction in the production of energy occurs. The cells of a living organism which are irradiated in lethal doses are not in a condition to carry out the oxidation of organic substances.

This, from our point of view, depends upon the fact that penetrating radiation directly destroys the metal-containing enzymes, and destroys the tissues' normal cycle of oxidation. An injury to the metal-containing enzymes is the result of a rupture of the haematinic portion



of the protein. I will allow myself to put forward certain general considerations as to the reasons why the metal-containing enzymes can be held to be substances of special radiosensitivity. Among such reasons is this, that the variable valency metal-containing enzymes, under the action of penetrating radiation, are easily subjected to oxidation, as a result of which a break in the bond between the haem and the protein may occur. Metals, in comparison with non-metallic substances entering into the composition of the cell, are capable, to a considerable extent, of absorbing the energy of the penetrating radiation and the absorbed radiation dose will be larger by several orders than in other organic compounds. It is also known that the metal-containing enzyme systems are concentrated in the structured inclusion bodies of cells and in connection with this they can be regarded as substances existing in cells in a more or less solid state. Investigations in the field of radiation chemistry have shown that as the concentration of the dissolved substances increases, there begins more and more to emerge a direct action of the penetrating radiation. In these conditions, the oxidation and reduction reactions are frequently accompanied by the destruction of organic substances. In this contribution I want to draw the attention of radiobiologists to the pressing problem of the need to study adequately the state of the metal-containing substances in the cell of an irradiated organism.

STOYANOVA: The method of the gas micro-chamber, developed as a result of the study of the interaction of electrons with biological preparations, allowed us to produce in the electron microscope conditions for the observation of wet biological objects and living micro-organisms, such that the latter remain capable of life. This provides us with the possibility of studying the dynamics of changes in the biological preparations under the action of various factors, including (since an electron beam is a source of ionizing radiation) ionizing radiation.

The maintenance by micro-organisms of their life potential is proved by the fact that those processes which took place prior to the moment of irradiation are continued in the cells. They continue to divide, grow and interact with each other.

The investigation of the action of ionizing radiations was carried out on living bacteria *B. mycoides* and *B. mesentericus*. Under direct observation in an electron microscope, the bacteria were examined from the beginning in their initial condition and then after the reaction. It was discovered that for radiation doses of up to  $10^6$  r for the first 5 to 10 sec no marked morphological variations are observed, and

certain processes are continued which were taking place in the cells prior to the moment of irradiation. With doses of  $10^7$  to  $10^8$  r for the first few seconds marked morphological injuries occur, the membrane and flagellae are noticeably destroyed and the protoplast is altered.

When investigating the action of ionizing radiation on collagen in the electron microscope in a moistened state, we managed to observe how, under the action of ionizing radiation, a breakdown occurs in certain interconnections in the collagen fibrils.

FRANK: The present section of the discussion has attracted the greatest attention. This is quite natural since during the last years the previously unknown submicroscopical cell structure was discovered and "super-molecular morphology" began to develop. For this reason it is of considerable interest to discuss the problem of the damaging effect not only from the point of view of the initial effect on different substances but also on this highly differentiated supermolecular organization.

Therefore one must agree with Dr. Alexander who paid attention to the possible primary role of damage to membrane organization (therefore function also) and probably to multi-membrane systems. Really, damage to such membrane molecular organization can have much more consequence than the inactivation of single, separately studied, biologically important molecules, particularly enzymes.

One must also agree with the point of view of Prof. A. M. Kuzin, who paid attention to the fact that the function of such membranous or multi-membranous systems can be damaged even by single cross-linkages, and also by the change in the activity of enzymes, not as of single molecules but as of the components of branched surface structures. I believe that everybody will agree with the fact, that when we transfer our interests from damage to substances to damage to cell organization we by no means oppose the one by the other. Here, in fact, a different interpretation of the acceleration mechanisms of primary lesions is given. The essence of this matter is not that chemically important molecules are inactivated, thus weakening one of the links in metabolism but, in fact, that single molecules which are the components of functional structural systems are inactivated, or the bonds between them are damaged. Here the inactivation of single molecules or damage to chemical bonds disturbs the function of the system in a similar way as, if we may use a rough analogy, one broken tooth only in a single gear-wheel can lead to the arrest of the whole mechanism.

Besides that we must note that structural disorganization (nobody has mentioned this directly during the discussion) influences primarily all the cellular mechanisms of autoregulation. Probably,

just multi-membranous systems as well as the whole submicroscopical architecture of the nucleus and cytoplasm are closely allied in the maintenance of the order of metabolic processes and self-adjustment to the most "beneficial conditions of work". We have no doubt of the connection between structural organization and function, but here my purpose is to emphasize a most important thing—connection with "control" of cell function. It is obvious that the disorganization of the structure caused by irradiation, must have the most distinct features of an accelerating mechanism, if it is connected with the disorganization of control. We drew these conclusions in our report (G. M. Frank, A. D. Suezdko) paying attention to the rhythmical processes and to the connection of this rhythm with the structural mobility. If one imagines, though hypothetically, that not only a definite organization is necessary, but also that continuous alteration of this organization with time is a part of the controlling mechanism, then, as a background to that system it is easier to appreciate the more pernicious role of structural damage, compared with a consideration of this structural damage only in statistical terms.

BACQ: VI. Is the oxygen effect universal? A part of the damage caused by ionizing radiation does not depend upon oxygen.

GRAY: In one sense the answer can be negative. Many of the reactions already investigated in regard to cell damage have been shown to occur when the irradiation takes place in the complete absence of oxygen. On the other hand, other injuries to cells and organisms are augmented in the presence of oxygen during irradiation. It is very important, however, to point out the nature of these injuries. The oxygen effect is observed when studying (a) injury to the chromosomes, (b) the inhibition of cell multiplication, (c) the frequency of mutation in micro-organisms, (d) the inhibition of the synthesis of nucleic acids, (e) the inhibition of the synthesis of adaptive enzymes, (f) the inhibition of the reproduction of viruses inside *baeilli*.

All these effects have common features, a study of which would help radiobiologists to look into this problem. All the effects mentioned above are in some way or another connected with the reduplication of genetic material inside the cell. When bacteria die, as a result of the introduction of radioactive phosphorus, the initial lesion occurs in the chromosomes; it is sensitive to oxygen concentration.

However, in regard to other physiological effects, in particular in regard to the permeability and passage of ions through the cellular membranes we have unfortunately very little information.

The release of potassium from the erythrocytes does not depend upon the concentration of oxygen. I would be very glad if the effect of oxygen could be studied for other processes as well.

BACQ: Certain effects which do not depend upon oxygen were pointed out by Hollaender, for example the inhibition of mitosis in the neural crest cells of the grasshopper. I myself have studied the effect in decreasing the resistance of the membranes of the skin. This effect has a momentary latent period of the order of one second.

Is there any other example of the independence of the radiation effect from oxygen?

EIDUS: I will quote facts concerning the question of the universality of the oxygen effect and the conditions for the destructive action of oxygen.

Some years ago we discovered that in one and the same protein molecule there arise under radiation two kinds of concealed injury, if we judge from the enzymatic activity.

Some concealed injuries are converted into manifest injuries under the action of heat, others under the action of oxygen. We suppose that in both cases the effect is connected with the conservation of a part of the absorbed energy in the long-lived excited state of the protein. This has been confirmed in our joint work with Kayushin.

It has been shown that the concealed injuries are connected with the long conservation of unpaired electrons in the protein which are even available in solution. Heating a solution of myosin to 20°C leads to the inactivation of the molecules and simultaneously to the disappearance of the unpaired electrons.

The action of heat and oxygen are independent, that is, both these agents act on different injuries.

It is interesting that Powers in his work on biological objects arrives at the same conclusions as to the existence of short- and long-lived excited states, which are divisible into oxygen-dependent and oxygen-independent as we concluded from our experiments with proteins; in addition, his division even compares quantitatively with ours. A comparison of the results of the experiments carried out by Powers on dry objects, and by us on solutions is possible. This will allow us to erect a bridge from our data to whole cells, to which our respected President referred at the first session.

The physical mechanism for damage both by oxygen and by heat is so far not known for certain. But there are facts which throw light on this question. An analysis of the literature and private data on the



nature of the results of the action of physical agents—heat, oxygen, nitric acid, water, etc.—on the ESR spectrum, on the one hand, and the radiosensitivity of various objects on the other, led us to the conclusion that the damaging action of oxygen is carried out only with the participation of a certain amount of water. We have shown experimentally that water is necessary even for the realization of concealed injuries, not connected with oxygen.

Pepsin, irradiated in dry form, contains concealed injuries which appear when it is heated in aqueous solution. However, when incubated in dry form, even at temperatures higher than  $100^{\circ}\text{C}$  it is impossible to uncover concealed injuries that are known to exist. Thus water is, in our opinion, a necessary participant for the realization of injuries which are both dependent upon, and independent of, oxygen.

In conclusion, I would like to point out that the existence of an oxygen after-effect in solutions must compel us to approach with great care the results of experiments carried out as it were anaerobically but when the result of the irradiation is already verified in the presence of oxygen.

MARKOVICH: One further example to complete the list given by Gray. Bacteriophage in cells up to the last moment of the latent period is not sensitive to oxygen; bacteriophage outside the cell is also not sensitive to oxygen.

PASSYNSKY: In work carried out with Pavlovskaya we reached the conclusion that under the direct action of radiation, the oxygen effect is governed by the transition of the irradiated oxygen into an excited state  $\text{O}_2^*$  (with an energy of 5 to 6 eV) or in the state of the molecular ion  $\text{O}_2^-$ , as a result of the absorption of an electron knocked out, for example, from the protein. In both the states mentioned, the oxygen can be an acceptor of hydrogen (or of protons) and this affects the redistribution of hydrogen ions in the protein molecule. In the indirect action of radiation (with a participation of molecules of the solvent) the oxygen effect, at least in regard to the oxidation of the SH groups, is quantitatively explained by the formation of  $\text{HO}_2$  radicals. It is necessary, however, to take into account the reaction  $\text{HO}_2 \rightarrow \text{H}^+ + \text{O}_2^-$ , which gives a second method for the formation of  $\text{O}_2^-$  together with the direct method  $\text{O}_2 + e \rightarrow \text{O}_2^-$ .

Thus, the influence of oxygen under various conditions of the action of the radiation may have common mechanisms. The influence of the oxygen following irradiation both upon the hydrogen bonds of proteins and nucleic acids, and also on the formation of new products of radio-



lysis of water (transition  $H \rightarrow HO_2$ ) has such an overall significance for any living cell that the oxygen effect from our point of view must be regarded as a universal factor in radiobiology.

BUDNITASKAYA: When discussing the question of the universality of the "oxygen effect", one should emphasize that many destructions of normal biochemical reactions under radiation action on the living organism are characterized by intensive oxidation. In connection with this the biochemical changes connected with the formation in the irradiated organism of lipid peroxides with the participation of lipoxidase are of great interest.

The enzymatic oxidation which is carried out by means of the peroxidase proceeds only with the formation of primary peroxides. Under these conditions, the action of the oxygen on the double bonds, through the mutual influence of the atoms of the intermediate free radicals and the formation of hydroperoxides, is strengthened under the influence of the radiation.

We have succeeded in showing that when the leaves of plants of various kinds are irradiated with doses of 1 to 20 kr their lipid content is altered very sharply. The chromatographic division of the fraction of free lipids from the irradiated and non-irradiated leaves, allowed us to establish the disappearance of certain unsaturated acids from the extract of free lipids of irradiated plants even within 15 min after irradiation. At the same time, we observed sharp changes in the content of lipid peroxides. In comparison with the non-irradiated leaves, the amount of peroxides in the fraction of free lipids in the irradiated plants increases even within 10 min of irradiation by approximately 2 to 6 times. But the irradiation of the leaves in which the enzyme, lipoxidase was inactivated by steam did not lead to an increase of the content of peroxides.

Consequently, the biological action of ionizing radiation on the plant is manifested in the activation of the enzyme system of the lipoxidase (by 30 to 50 per cent) during the first 24 hr after irradiation which oxidizes by means of molecular oxygen the unsaturated fatty acids, thus strengthening the "oxygen effect" *in vivo*.

BACQ: VII. Concerning the mechanism of action of protective substances.

The chemical protective substances have various mechanisms of operation which can be simultaneous or synergetic. Each protective system must be examined separately.

PASSYNSKY: In connection with the formulation of the problem under discussion, I would like to point out the comparatively simple dependence which is characteristic of the action of thiol compounds.

When thiols are oxidized by ferrieyanide, cysteine-oxidase, or penicillin (which, as we have shown, is also an oxidizing agent with  $\epsilon'_0 = +0.3$  V), one can establish the following sequence of thiol compounds in terms of the ease of oxidation.

I	>	II	>	III
Cysteine		Glutathione		Methionine
Cysteine methyl ester		Thioglycolic acid		S-Methyleysteine
		Thiolactic acid		Cystine
Cysteine ethyl ester		N-Acetylcysteine		Serine

The protective properties of these thiols are distributed in the same way. The redox potentials of the compounds of groups I and II are close ( $\epsilon'_0 \approx 0.06$  to  $0.08$  V) so that the similarity of oxidation of the SH-groups in these compounds depends upon the degree of polarity of the SH-group, or the proportion of the ion composition in this link— $S^- \dots H^+$ . The higher the polarization, the stronger is the form— $S^-$ , presented, the higher the reaction capacity of the thiols.

In the compounds of group I, the possibility exists of transferring a proton from  $-SH$  to the  $-NH_2$  group with the formation of the dipolar form  $-NH_3^+$  and  $-S^-$  similar to the zwitterion form  $^+NH_3-R-COO^-$  of the amino acids.

Group II contains compounds in which this proton transition is, for various reasons, complicated: in glutathione by the distance of the  $-NH_2$  from the  $-SH$ , in acetylcysteine (the acetyl substituent in the  $-NH_2$  group, in the thiols and thiolactic acids) by the absence of the  $-NH_2$  group.

Finally, in Group III compounds proton transference is in general absent in view of the replacement of the hydrogen of the  $-SH$  groups in methionine, S-methyleysteine and cystine and in serine—as a result of the fact that the  $-OH$  link has energy of 110 kcal, against 82 kcal in the  $-SH$  group and the expulsion of the hydrogen atom is much more difficult. In the organism, however, the methionine and the cystine may produce some action due to the partial metabolic transition to cysteine.

If we compare cysteine  $HS-CH_2-CH < \begin{smallmatrix} NH_2 \\ COOH \end{smallmatrix}$  with the best defence substance, cysteamine  $HS-CH_2-CH_2-NH_2$ , then we may see that in the latter the transition of the proton with the formation of a di-

polar form  $S^- - CH_2 - CH_2 - NH_3^+$  may be still more sharply expressed than in cysteine due to the absence around the  $-NH_2$  group of the carboxyl group, which weakens the transfer.

Thus there exists a completely clear sequence of reactions of all these compounds depending upon the degree of ionization and the degree of polarization of the sulphydryl group. The protective capacity of the thiols proves to be greater the higher the proportion of the ion state in the sulphydryl group.

One can point to two experimental methods for establishing the di-polar or zwitterion form of the cysteamine and allied compounds: (1) the high values of the dipole moment and the dielectric increment ( $d\epsilon/dc$ ) in solution; (2) the analysis of the curves of the potentiometric titration.

What has been said does not of course constitute an overall theory but only a partial regularity which embraces, however, quite a large number of thiol compounds. I would like once more to emphasize that this regularity has a general significance, not only for the protective action in regard to radiation, but also for many other oxidizing actions on thiol compounds.

BACQ: I would like to stress that the data quoted by Passynsky are in complete agreement with the work of our laboratory.

BARENDSEN: I would like to give additional information in regard to the protective properties of cysteamine.

Foch, the Professor of the Medical-Biological Laboratory at Rijswijk, has shown that cysteamine is a good protective substance for mammalian cells in tissue culture.

But I have discovered that under  $\alpha$ -radiation the defensive effect of cysteamine is insignificant. In this case, the addition of cysteamine recalls the effect of anoxia. I do not think that both mechanisms—the action of anoxia and the defensive properties of the cysteamine are identical, but in both cases the effect of the injury is removed. In the case of  $\alpha$ -radiation, because of the high ionization density, considerable damage nevertheless occurs which does not allow the protective effect of the cysteamine to emerge to a sufficient extent.

BACQ: The action of  $\alpha$ -irradiation on the growing rootlets of an onion also varies only weakly in the presence of cysteamine. It has been shown in mice that the effect of the action of neutrons is also only insignificantly lessened under the influence of cysteamine even in comparison with the effect of the action of X-rays. Evidently this is a general phenomenon.

DUBININ: The study of the chemical protective agents can be considered in the analysis of the nature of the primary effects of radiation upon genetic material. In the work of Dubinin, Sidorov and Sokolov, this method was applied to living cells. By utilizing the phenol-ascorbic acid reaction with hydrogen peroxide, we have obtained free OH radicals chemically within the nucleus of the living cell. These radicals have given rise to a substantial genetic effect. In the nuclei of the cells of onion rootlets about 20 per cent of chromosome arrangements were found. In earlier work on the production of chromosome arrangements by visible light Dubinin, Sidorov and Sokolov showed that the iodide ion and certain other substances protect the nucleus from damage by the photodynamic process. We have used this protection to study its influence on the genetic effect from chemically derived radicals. By introducing into the cell ions of iodide, bromide and hydroquinone, quinone, hyposulphite, and thiourea, we have discovered that all these protect the chromosomes from chemically derived radicals.

However, it was discovered from experiments with X-irradiation that iodide and bromide ions have no defensive effect. Dury and Kalam in their paper proved that iodide ions penetrate the nucleus. Thus, the interception by iodide and bromide ions of the free radicals which arise in the radiolysis of water does not protect the chromosome from the effect of radiation. This points to the predominance of the direct action of the radiation on the chromosomes. Mekshenkov in our laboratories, while studying the effect of radiation on DNA solutions of various concentrations, showed that for low concentrations, when the indirect effect of the radiation prevails, the iodide and bromide ions provide a protection close to 100 per cent and in solutions of high DNA concentration, these ions do not protect the number of DNA molecules being measured.

This experimental analysis confirms the correctness of the forecasts made by many authors concerning the enormous significance of the direct action of the radiation on chromosomes. The experiments of Alexander carried out with dry polymers have great value. It follows from his data that for DNA molecules in chromosomes, one must expect the predominance of the direct radiation effect.

All this shows that when working out the problem of the chemical protection of genetic material, one must take into account, as a main factor, the direct effect of the radiation and not the reactions for competition for the free radicals.

TARUSOV: In the survey article by Rachinsky and Mozzhukhin in



which they examined the physico-chemical properties of hundreds of protective substances, the thesis is proposed that not every anti-oxidant is a protective substance. However, not one such protective substance is known as yet which does not possess anti-oxidant properties. We must recognize that all the existing defence substances are inhibitors of chain oxidation reactions, and the restricted nature of their action is explained by the presence of non-oxidizing primary reactions. In order to have total protection, it is necessary to seek certain other substances which are not oxidation inhibitors.

ZELENY: In the systems of connective tissue investigated by Brinkman the oxygen pressure was measured and its protective action shown. It seems obvious that this is explained by the interception of the H radicals, because cysteamine also has a protective action in these systems; I would like Baq to give us his views on the nature of the action of cysteamine in this tissue.

BAQ: The action of the cysteamine in these systems does not depend upon the oxygen tension. Disulphide derivatives and tryptamine which do not contain  $-SH$  groups and even sulphur have an analogous effect. It is possible that polysaccharides or hyaluronic acid in the connective tissue is transformed under the action of radiation to an excited state, and in the presence of a hydrogen donor is returned to the original condition.

SEMENOV: The damage to the bone-marrow, the intestines, the skin and other proliferating organs of mammals given doses of 200 to 700 r may not involve the mechanism of cellular death. In this case, the cessation of biosynthesis and the gradual depletion of the materials accumulated for the reproduction of the cells is of fundamental significance.

In this case, when carrying out investigations, it was necessary to examine how the protective substances affect the basal cells and not the differentiated cells such as leucocytes, lymphocytes, reticular cells in the bone-marrow, etc.

Thus what is of interest is not the action of the protective substances on the so-called radiosensitive cells but the action on the insensitive cells, which are the source of the production of the sensitive cellular elements. When examining the mechanism of the local protective effect, this possibility must be taken into account.

ROMANTSEV: The concept that has the greatest experimental foundation at the present time, from our point of view, is that concerning the mechanism of action of chemical protective agents according to



which the protective substances reduce the possibility of the formation of radicals at the instant of the action of X- and  $\gamma$ -rays. This notion of the mechanism of the action of the protective substances has a direct link with the oxygen effect.

It would, of course, be a mistake to connect the mechanism of the protective action of diverse compounds only with those of other variations of the oxygen concentration in the medium or the substrate. It is important, however, to emphasize that for all the means of chemical protection studied in experiments on mammals we succeeded in establishing the link with the oxygen effect.

This link was expressed to a greater or lesser extent for the protective substances belonging to the various classes of chemical compound. Of course, the alteration in the free oxygen concentration in different tissues was effected by means of various biochemical and physiological mechanisms.

For protective substances belonging to the group of cyanide compounds or to the nitriles or azides, the connection with the oxygen effect emerges in more open form. For amines which do not contain an  $-SH$  group and for aminothiols this connection with the oxygen effect frequently emerges in veiled form.

In technically exquisite experiments van Muir and van Bekkum have recently shown convincingly with the help of an oxygen electrode that the so-called biogenic amines—histamine, tryptamine, phenylethylamine,  $\beta$ -epinephrine—clearly reduce the concentration of oxygen in the spleen. There is a good positive correlation between the tissue hypoxia produced in the spleen and the prophylactic action of these amines.

In our experiments carried out with aminothiols we discovered that, when introducing such aminothiols as  $\beta$ -mercaptoethylmine,  $\beta$ -mercaptopropylamine, AET (aminoethylisothiuronium bromide), rats begin to consume a reduced amount of oxygen and the irradiation of the animals proceeds against the background of a general reduction in the intensity of oxidation-reduction processes. By making use of the polarographic method we have succeeded in showing that after introducing  $\beta$ -mercaptopropylamine in rabbits, the concentration of the free oxygen in the cortex of the brain is distinctly reduced.

Thus, one may regard as established the role of the oxygen effect in the mechanism of the action of aminothiols and biogenic amines in mammals.

One of the possible release mechanisms for radiation damage is the chain reaction evidently induced by various compounds, for example by organic peroxides in the lipid fraction. This concept has been

successfully developed by Tarusov. It is quite clear that the formation of the peroxides under irradiation conditions must be in direct dependence upon the concentration of free oxygen in the medium.

In fact it was discovered that after irradiating mice for 10 sec with a lethal dose of X-rays (13 kr) a certain increase in the amount of peroxide-like compounds in comparison with the control takes place directly after irradiation. It is important to note that the various protective substances, even including aminothiols, sharply reduce the output of the organic peroxides both in irradiated and in healthy animals.

It was also shown by these experiments that the oxygen effect is of tremendous significance for an understanding of the action of the protective substances belonging to the various classes of chemical compounds, including the aminothiols.

In the concluding speeches, Z. M. Bacq (Belgium), A. Hollaender (U.S.A.) and G. M. Frank (U.S.S.R.) referred to the successful organization and fruitful work of the symposium, and expressed their gratitude to its participants and organizers.











